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THE UNIVERSITY OF ALBERTA

STUDIES ON AN ACTIN-LIKE PROTEIN FROM CHICKEN PECTORALIS MUSCLE

by

KEVIN SWALLOW

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF Master of Science

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Spring 1988

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.....

Supervisor

.....

.....

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ABSTRACT

Breakdown of the Z line during meat ageing is believed to be a major contributing factor in the conversion of muscle to meat. An actin-like protein was isolated from the Z disc of chicken pectoralis muscle that differed from thin filament (F) actin in its solubility in a 0.6 Molar potassium iodide solution. Research was undertaken to determine if this protein is structurally different from thin filament actin. The UV spectra of F actin revealed that this protein contained a compound absorbing at 260 nanometres, presumably ADP, while Z disc actin did not. Removal of this compound could be accomplished by exhaustive dialysis or denaturation in 6 Molar urea. After removal of this compound, analysis of the F actin and Z actin samples was undertaken using high performance liquid chromatography and polyacrylamide gel electrophoresis. Loss of the 260 nanometre absorbing compound from thin filament actin caused this protein to exhibit identical behaviour to Z actin in the presence of denaturing salts and myosin. The removal of the 260 nanometre absorbing compound from F actin probably alters its conformation, producing a protein with a similar tertiary structure to Z actin. Evidence brought forth here suggests that the 0.6M KI insoluble actin present in the Z line of myofibrils is thin filament actin with a different *in situ* conformation. The possibility exists that this structurally different actin in the Z line may play a role in the conversion of muscle to meat.

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There are several people that have aided me along the rocky path of research in the last two years. Foremost is Dr. Fred Wolfe; his patience, understanding and assistance allowed me to pursue an area of research which was extremely interesting and informative. He provided guidance when needed, but also allowed me to wander through areas which were of specific interest to me.

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TABLE OF CONTENTS

Chapter	Description	Page
	ABSTRACT	iv
	ACKNOWLEDGEMENTS	v
	List of tables	x
	List of figures	xi
	List of photograghic plates	xiii
1	**INTRODUCTION**	1
	OVERVIEW OF MUSCLE STRUCTURE	1
	Structure and Composition of the thin filaments	5
	Structure and Composition of the thick filaments	8
	Structure and Composition of the Z line	10
	Alpha-Actinin	12
	Actin-like Protein of the Z disc	12
	Cytoskeletal Proteins	13
	MEAT AGEING	14
	Postmortem Metabolism	15
	Breakdown of Stromal Proteins During Meat Ageing	17
	Breakdown of Cytoskeletal Proteins During Meat Ageing	17
	Breakdown of Myofibrillar Proteins During Meat Ageing	18
	Breakdown of the Z Line During Meat Ageing	19

Chapter	Description	Page
	PROTEOLYTIC ENZYMES AND THEIR EFFECT UPON THE RESOLUTION OF RIGOUR MORTIS	20
	Alkaline Proteinase	21
	ATP Dependent Proteinase	21
	Neutral Proteinase	22
	High Molecular Weight Cytosolic Proteinase	22
	Calcium Activated Neutral Proteinase	23
	Lysosomal Proteinases	25
	SIMILARITIES AND DIFFERENCES BETWEEN Z DISC ACTIN AND THIN FILAMENT ACTIN	27
	STATEMENT OF THE PROBLEM	28
2	MATERIALS AND METHODS	30
	Protein Determination	30
	Protein Concentrating	31
	Polyacrylamide Gel Electrophoresis	31
	Muscle Tissue Preparation	31
	Z DISC ACTIN PURIFICATION	32
	Myofibril Preparation	32
	Extraction of Z Disc Actin from Myofibrils	33
	THIN FILAMENT ACTIN PURIFICATION	34
	Muscle Acetone Powder Preparation	34
	Extraction of Thin Filament Actin from MAP	36
	MYOSIN PURIFICATION	37
	ALPHA-ACTININ PURIFICATION	39

Chapter	Description	Page
	SCANNING ULTRAVIOLET SPECTROPHOTOMETRY	41
	Enzymatic Digestion of Z Disc Actin and Thin Filament Actin	43
	HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF THE CHYMOTRYPTIC DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN.....	43
	Denaturation of Z Disc Actin and Thin Filament Actin	44
	AGGREGATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN	44
	SUPERPRECIPITATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN IN THE PRESENCE OF MYOSIN	45
	ELECTRON MICROSCOPY	45
	Muscle Bundle Preparation	45
	Sample Preparation for Electron Microscopy	45
	SDS-PAGE ANALYSIS OF THE CANP DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN	46
3	RESULTS	48
	SCANNING ULTRAVIOLET SPECTROPHOTOMETRY	48
	HPLC ANALYSIS OF THE CHYMOTRYPTIC DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN	51
	AGGREGATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN	54
	SUPERPRECIPITATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN IN THE PRESENCE OF MYOSIN	63
	ELECTRON MICROSCOPY	67
	Removal of the Z Lines	67
	Reconstitution of the Z Lines	68

Chapter	Description	Page
	SDS-PAGE ANALYSIS OF THE CANP DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN	73
4	DISCUSSION	76
5	CONCLUSION	88
	BIBLIOGRAPHY	90
	APPENDICES	
	1. Polyacrylamide Gel Electrophoresis	98
	2. Buffers and Their Chemical Composition	102
	3. Enzyme Activity	104

LIST OF TABLES

Table	Description	Page
1	Proteinases in muscle tissue	20
2	Proteins used in the attempted reconstitution of the Z line of myofibrils	73

LIST OF FIGURES

Figure	Description	Page
1	Schematic representation of the organization and major constituents comprizing skeletal muscle	3
2	Schematic representation of a thin filament in a skeletal myofibril	7
3a	Schematic representation of a thick filament in a skeletal myofibril	9
3b	Schematic representation of the structure of a myosin molecule showing the 2 trypsin sensitive cleavage sites	9
4	Schematic representation of the Z line of a skeletal myofibril	11
5	Scanning UV spectrophotometric analysis of freshly purified Z actin and F actin	49
6	Scanning UV spectrophotometric analysis of F actin using Z actin as the blank	50
7	Scanning UV spectrophotometric analysis of F actin and Z actin denatured in 6M urea	52
8	Scanning UV spectrophotometric analysis of F actin and Z actin exhaustively dialyzed in a benign buffer	53
9	HPLC analysis of the 1 hour chymotryptic digest of Z actin and F actin at 210 nm	55
10	HPLC analysis of the 1 hour chymotryptic digest of Z actin and F actin at 280 nm	56
11	HPLC analysis of the 22 hour chymotryptic digest of Z actin and F actin at 210 nm	57
12	HPLC analysis of the 22 hour chymotryptic digest of Z actin and F actin at 280 nm	58
13	Aggregation of freshly purified Z and F actin upon addition of $CaCl_2$ and ATP	60
14	Aggregation of denatured Z and F actin in the presence of $CaCl_2$ and ATP	61

Figure	Description	Page
15	Aggregation of dialyzed Z actin and F actin in the presence of $CaCl_2$ and ATP	62
16	Superprecipitation of freshly purified F actin and Z actin in the presence of myosin	64
17	Superprecipitation of denatured F actin and Z actin in the presence of myosin	65
18	Superprecipitation of F actin and Z actin after dialysis in a benign buffer in the presence of myosin	66
19	Flow chart illustrating the possible relationship between Z actin and F actin.....	81

LIST OF PHOTOGRAPHIC PLATES

Plate	Description	Page
1	Electron micrograph of a segment of a skeletal myofibril showing the Z lines, I bands, A bands and the H zone	4
2	SDS-PAGE illustrating the steps involved in the preparation of myofibrils and isolation of Z actin	35
3	SDS-PAGE illustrating the steps involved in the purification of thin filament (F) actin from muscle acetone powder (MAP)	38
4	SDS-PAGE illustrating the steps involved in the purification of myosin	40
5	SDS-PAGE illustrating the steps involved in the purification of alpha-actinin	42
6	Electron micrograph of a myofibril with its ultrastructure intact	69
7	Electron micrograph of a myofibril treated with CANP and 10mM $CaCl_2$	70
8	Electron micrograph of a myofibril treated with CANP and 10mM $CaCl_2$	71
9	SDS-PAGE illustrating the various proteins isolated for the attempted reconstitution of the Z line in myofibrils	72
10	SDS-PAGE demonstrating the effect of the addition of CANP and 6mM $CaCl_2$ to various samples of F actin and Z actin	75

INTRODUCTION

A complex array of interdependent physiological and biochememical events occur during the period of carcass conditioning which results in the conversion of muscle to meat. After stunning, exsanguination and circulatory failure cause the oxygen supply to the muscle cell to cease. The absence of oxygen forces the cell to regenerate ATP via anaerobic glycolysis, which leads to the formation of lactic acid and a corresponding drop in pH. Eventually anaerobic glycolysis ceases, resulting in the fall of adenosine triphosphate (ATP) levels and the rise of calcium ion levels. These two factors lead to the stiffening of muscle called rigour mortis. Continued ageing of the carcass brings about the resolution of rigour mortis, leading to an increase in meat tenderness. Although our knowledge of these changes during meat ageing has increased considerably in recent times, many aspects of this transformation are still unclear. Before examination of the factors involved in the conversion of muscle to meat, a brief review of muscle structure is required.

OVERVIEW OF MUSCLE STRUCTURE

The majority of meat foods originate from striated skeletal muscle. Therefore, this review will focus primarily on striated muscle. Individual skeletal muscles are surrounded by a thin sheath of connective tissue called the epimysium. Each muscle is comprised of many columns of tissue called fasicles or bundles, which are covered by a sheath of connective tissue called the perimysium. The perimysium and epimysium are primarily made up of collagen. The muscle fasicles are

composed of muscle cells called myofibres which are surrounded by a connective tissue sheath called the endomysium. The endomysium is primarily made up of very small fibres of collagen called reticular fibres. Together, these layers of connective tissue form a continuous meshwork in and around the muscle which strengthens it and aids in attachment of muscle to bone.

Myofibres are slender, multinucleated cells with a diameter varying between 10 and 100 μm and length from several mm to more than 30 cm, depending on the muscle (Lockhart, 1972). The muscle cells are comprised mainly of thread-like structures called myofibrils. When viewed in a microscope, these myofibrils appear striped or striated due to the presence of alternating light and dark bands. In a polarizing microscope, the bright areas are called the anisotropic or A bands because they have the ability to rotate polarized light. Conversely, the dark regions do not have this ability, and are called isotropic or I bands. However in nonpolarizing microscopes, I bands are light and A bands appear dark. The I bands have a thin line bisecting them which is known as the Z line or Z disc. Figure 1 illustrates the gross ultrastructure of skeletal muscle. The repeating portion of the myofibril between two adjacent Z lines is called a sarcomere and is the smallest unit capable of contraction. Each A band contains a lighter region in the centre which is called the H zone. Bisecting this H zone is a thin dark area known as the M line. Plate 1 is an electron micrograph of chicken pectoral muscle illustrating the structure of a myofibril. The A band is composed primarily of thick filaments while the I band is made up of thin filaments.

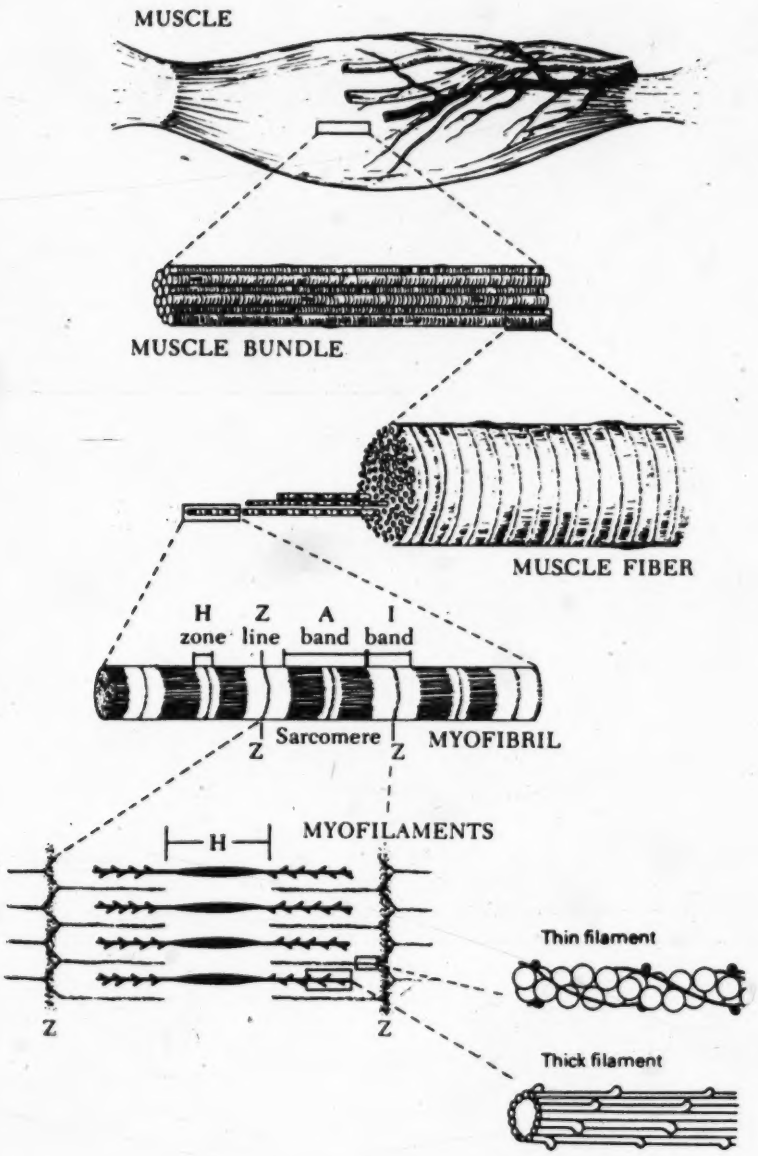

Figure 1. Schematic representation of the organization and major constituents comprizing skeletal muscle (Modified from W. Bloom and D.W. Fawcett, A Textbook of Histology, 9th ed., 1968, p. 273. Reprinted by permission of W.B. Saunders Company, West Washington Square, Philadelphia, Pa.).

Plate 1. Electron micrograph of a segment of a skeletal myofibril showing the Z lines, I bands, A bands and the H zone. The portion of a myofibril from one Z line to the next is called a sarcomere. (magnification = 31,000x)

The thick filaments have a diameter of 14 to 16 nm while the thin filaments have a diameter of 6 nm (Huxley, 1957).

Structure and Composition of the Thin Filaments

There are numerous structural and regulatory proteins associated with the thin filaments. The major protein in the thin filaments is actin, which constitutes about 25 percent of the protein in myofibrils (Ebashi and Nonomura, 1973). Monomeric globular (G) actin has a molecular weight (MW) near 42,000 Daltons as determined by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and amino acid sequencing (Elzinga et al., 1973). In muscle cells, G actin occurs in polymers which form alpha helical, 2 stranded filaments containing 300 to 800 G actin monomers (Murray and Weber, 1974). In vitro, G actin polymerization can be induced in a solution containing 0.1M KCl, 2mM $MgCl_2$ and 1mM ATP, buffered at pH 8.0. It is thought that the presence of the cations screens out the negative electrostatic charges on the actin monomers, allowing them to closely associate (Golaszlioska et al., 1978). The energy required for polymerization is supplied by the hydrolysis of ATP yielding adenosine diphosphate (ADP) and inorganic phosphate. ADP remains stoichiometrically bound to each G actin monomer in the F actin filament while the inorganic phosphate is released.

Several proteins have been isolated from skeletal muscle which have the ability to bind to actin. Tropomyosin is a 100% alpha helical protein consisting of 2 subunits of 34,000 Daltons which coil around each other (Cohen and Holmes, 1963). Tropomyosin fits in the groove between the two helical chains in the F actin filament and, in conjunc-

tion with the troponin complex, functions in regulation of muscle contraction.

A second major regulatory protein in the thin filament is troponin. This protein has a molecular weight of 76,000 Daltons and is made up of three subunits. One subunit, troponin C (TnC), has a high affinity calcium binding site. When calcium is not bound at this site, a second troponin subunit, known as TnI, inhibits the interaction of actin and myosin. However, if the Ca^{++} concentration reaches 10^{-5} M, this ion will bind to TnC in sufficient amount to cause a conformational change to occur in the troponin complex. This change causes tropomyosin to swing out of the path between actin and myosin. With the steric hindrance due to the presence of tropomyosin removed, actin and myosin are able to interact. If ATP is present, shortening of the sarcomere and muscle contraction takes place. The energy for muscle contraction is provided by ATP hydrolysis and released by the enzyme myosin ATPase, part of the myosin molecule. The last subunit of troponin, TnT, binds the other two troponin subunits to tropomyosin and the actin filament. Together, the three subunits interact with actin, tropomyosin and calcium ions to regulate muscle contraction (Mannherz and Goody, 1976). Figure 2 represents the structure of thin filaments in muscle cells.

Several other actin binding proteins have been isolated from skeletal muscle cells. These proteins perform various functions in the cell (for a review see Stossel et al., 1985). For example, profilin binds to G actin at a ratio of 1:1, preventing its polymerization. Gelsolin binds to F actin in the presence of calcium causing the fila-

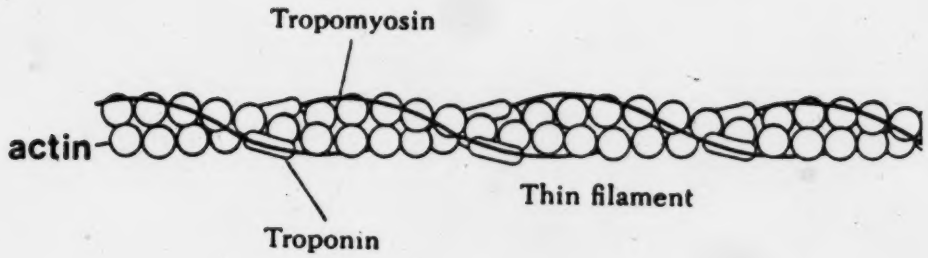

Figure 2. Schematic representation of a thin filament in a skeletal myofibril (Modified from J. Murray and A. Weber, "The Cooperative Action of Muscle Proteins," 1974, p. 60. Scientific American, Inc. All rights reserved).

ment to be cleaved at that site. Although these proteins serve vital roles in the muscle cell, they are thought to have little influence on postmortem muscle conditioning and thus will not be covered here.

Structure and Composition of the Thick Filaments

The major protein of the thick filament is myosin, which contributes 55-60% to the total myofibrillar protein (Ebashi and Nonomura, 1973). Figure 3a illustrates the assembly of myosin molecules in the thick filaments. Monomeric myosin has a molecular weight of ~480,000 Daltons and is made up of 2 subunits called heavy chains and 4 smaller subunits called light chains. Each heavy chain has a MW of ~200,000 Daltons while the MW of the light chains varies from 16,000 to 27,500 Daltons depending on the myosin source (Weeds, 1980). Myosin has a trypsin sensitive cleavage site which breaks the molecule into 2 parts; light meromyosin (LMM) and heavy meromyosin (HMM). Figure 3b illustrates the tryptic hydrolysis fragments of myosin. LMM is the tail region of the myosin molecule and has a molecular weight of ~150,000 Daltons. HMM can be further cleaved by trypsin into 2 fragments; S1 and S2. The S1 fragments consist of the 2 myosin globular heads, each having a MW of ~115,000 Daltons. This region contains the myosin ATPase enzyme. The S1 fragment is made up of a tail region, much like LMM, and has a MW of ~100,000 Daltons. The part of the myosin molecule that is susceptible to tryptic hydrolysis is believed to be the region that flexes or hinges during muscle contraction (Harrington, 1971). The myosin molecules in the thick filament are polarized, that is they are arranged so that half of the HMM heads face one Z line while the

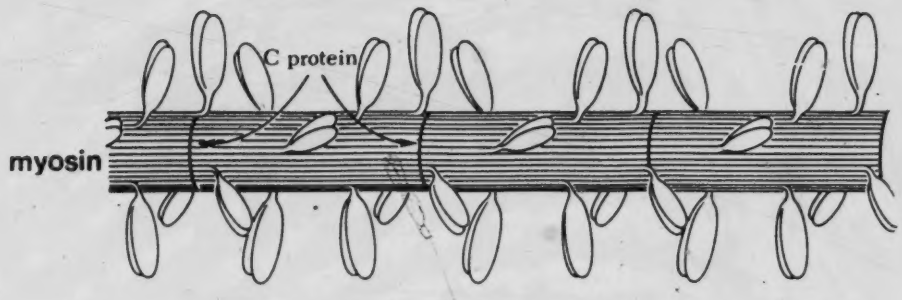

Figure 3a. Schematic representation of a thick filament in a skeletal myofibril (Modified from J. Murray and A. Weber, "The Cooperative Action of Muscle Proteins," 1974, p. 61. Scientific American, Inc. All rights reserved).

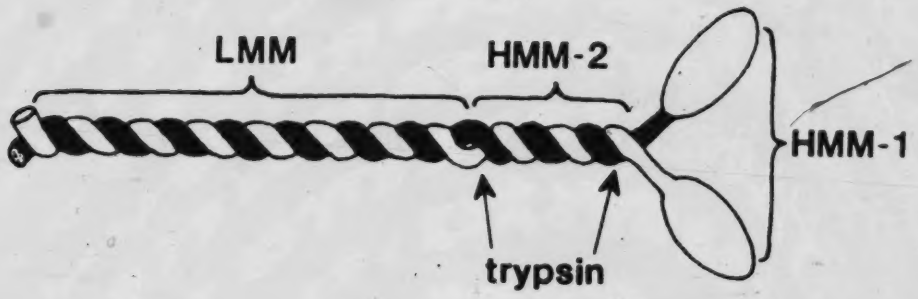

Figure 3b. Schematic representation of the structure of a myosin molecule showing the 2 trypsin sensitive cleavage sites. LMM (light meromyosin); HMM-1 (heavy meromyosin, S-1 subunits); HMM-2 (heavy meromyosin, S-2 subunit). (Modified from H.J. Swatland, Structure and Development of Meat Animals, 1984, p. 162. Reprinted by permission of Prentice-Hall, Inc., Englewood Cliffs, New Jersey).

remainder face the Z line on the opposite side of the sarcomere. Because of this arrangement, the middle segment of the thick filaments contain no HMM heads and, therefore cannot interact with actin. This region of the thick filaments is called the H zone. The H zone is believed to contain the myosin binding proteins myomesin, creatine kinase and M protein (Trinick and Lowey, 1977). These proteins are thought to join the adjacent myosin molecules in the lateral arrangement of thick filaments. Another protein believed to play a role in the integrity of the thick filaments is C protein. C protein is thought to bind the thick filaments together in seven places on either side of the M lines at intervals of 43 nm (Offer et al., 1973). These researchers surmised that C protein maintains the diameter of the thick filaments by allowing only 12 to 20 myosin rods to pack together in any one location. Several hundred myosin molecules make up one thick filament, which is about 1.5 μm in length.

Structure and Composition of the Z Line

The Z line is the functional unit in myofibrils which connects one sarcomere to the next. In human skeletal muscle its width varies from 60-70 nm in white fibres to 110-140 nm in red fibres (Sjostrom et al., 1982). However, in insect flight muscle the Z line may have a width up to 140-165 nm (Bullard and Sainsbury, 1977). Since the discovery of the Z line, numerous workers have made suggestions regarding its ultrastructure (Ashhurst, 1967; Katchburian et al., 1973; Saide and Ulbrick, 1974; Ulbrick et al., 1977; Granger and Lazarides, 1979a). Although the exact structure is still not known, Figure 4 is a schematic

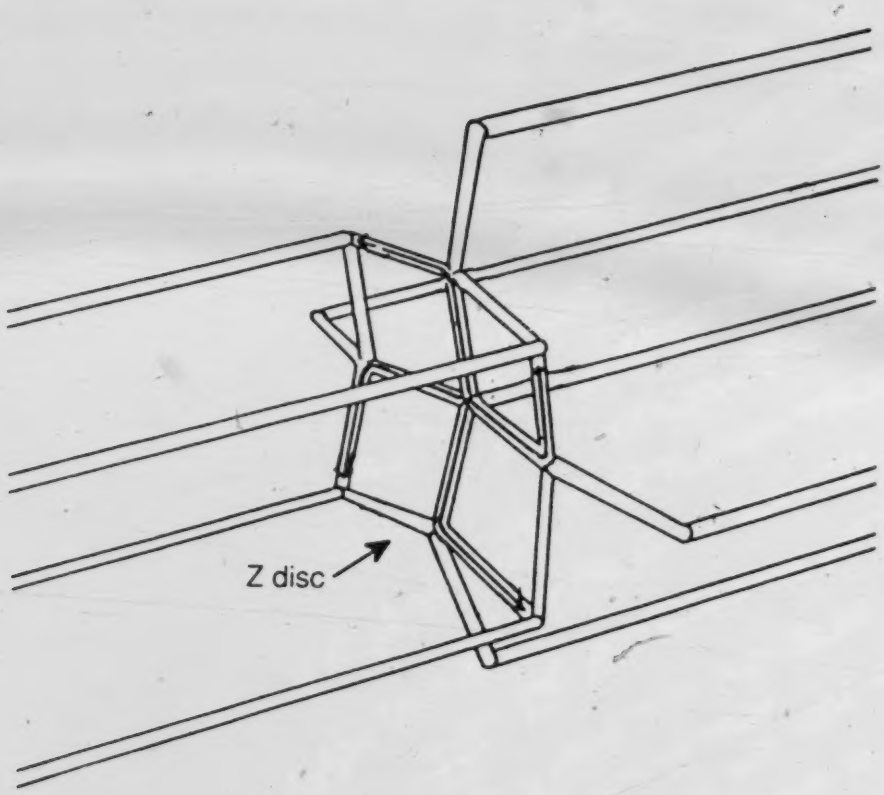

Figure 4. Schematic representation of the Z line of a skeletal myofibril. It is thought that thin filament actin from adjacent sarcomeres is joined in the region of the Z disc by proteins such as alpha-actinin. Z disc actin, desmin and vimentin (Modified from R.M. Robson, et al., "Biochemistry and Molecular Architecture of Muscle Cell 10-nm Filaments and Z-line: Roles of Desmin and Alpha-Actinin," 1981, p. 6 in 34th Annual Reciprocal Meat Conference Proceedings. Reprinted by permission of the National Live Stock and Meat Board, 444 North Michigan Avenue, Chicago, Illinois).

diagram of the Z line which seems consistent with the bulk of the data presented to date. This representation shows the staggered arrangement of thin filaments entering the Z line from opposite directions. Although the composition of the Z line is not entirely known, several proteins, including alpha-actinin, actin-like proteins and cytoskeletal proteins, are believed to play a role in maintaining its integrity.

Alpha-Actinin

One of the proteins thought to be essential for maintaining the structure of the Z line is alpha-actinin (Suzuki et al., 1976). With the aid of immunofluorescent microscopy, Lane et al., (1977) revealed that alpha-actinin is present in the Z line. This protein has a molecular weight of ~206,00 Daltons, consisting of two subunits of ~100,000 Daltons each. Alpha-actinin has the ability to induce the polymerization of G actin to F actin (Maruyama and Ebashi, 1965). Although the function of alpha-actinin is not fully understood, it is known that this protein can cause actin filaments to become arranged in a parallel fashion by binding to the end of F actin *in vitro* (Suzuki et al., 1975). Because of this ability, these researchers suggested that alpha-actinin may bind the actin filaments to the Z line *in vivo*.

Actin-like Protein of the Z disc

Gard and Lazarides (1979) discovered an actin-like protein in the Z line which differed from thin filament actin in that it is insoluble in 0.6 M KI. Taking this one step further, Nagainis and Wolfe (1982) suggested that this Z disc actin may be a structural protein in

the Z line. These researchers proposed that alpha-actinin functions to bind the thin filaments to the Z disc actin. It has been shown that treatment of intact myofibrils with an endogenous proteolytic enzyme from skeletal muscle, now known as calcium activated neutral proteinase (CANP) or Calpain, breaks down the Z line and releases alpha-actinin into the supernatant (Reddy et al., 1975; Suzuki, 1975; Dayton et al., 1976). Nagainis and Wolfe (1982) also demonstrated that CANP has the ability to hydrolyze Z disc actin, but not alpha-actinin or thin filament actin. The possibility exists, therefore, that this Z disc actin may be an important structural component of the Z line.

Cytoskeletal Proteins

Evidence suggests that other proteins, in addition to actin, myosin and alpha-actinin, may be partially responsible for sustaining the mechanical properties of the muscle cell. These cytoskeletal proteins are thought to form thin fibres in the myofibril, possibly accounting for some of the structural integrity of skeletal muscle (Granger and Lazarides, 1979b; Robson et al., 1981). The fibres are called intermediate filaments because they are thought to have diameters of ~10 nm, which is about half way between the diameters of thick and thin filaments (Ishikawa et al., 1968). Intermediate filament proteins, including desmin, filamin, vimentin and connectin, have been identified at or near the Z lines of myofibrils (Robson et al., 1981; Bechtel, 1979). Researchers have speculated that such intermediate filaments form a scaffold-like structure which may link adjacent sarcomeres in the region of the Z line (Huiatt et al., 1980; Granger and Lazarides,

1979b). Using immunofluorescent phase contrast microscopy, Lazarides and Hubbard (1976) and Granger and Lazarides (1979b) demonstrated that desmin and vimentin are located in the periphery of the Z line. They suggested that these proteins may function in aligning sarcomeres and consolidating the myofibrils. Although the extent of their involvement in the routine functioning of myofibres is not known for certain, it is probable that the cytoskeletal filaments are important in maintaining cell integrity in the resting and contracting states.

The foregoing presented a summary of the major and minor known myofibrillar proteins. These proteins serve various roles ranging from maintenance of cell integrity to regulation of muscle contraction. As the function of these and perhaps other muscle proteins becomes better known, it will be possible to explain more of the many complex events that bring about the conversion of muscle to meat.

MEAT AGEING

Muscle cells do not cease to operate when blood flow stops. Rather, the myofibres continue to function, albeit in a limited fashion, for many hours after the death of the animal. Necrobiosis is the term used to describe independent cellular activity during the postmortem period. It is during this period and beyond that muscle tissue undergoes the changes which convert muscle to meat, and lead to the development of flavour and tenderness.

Postmortem Metabolism

After stunning and exsanguination, oxygen transport to the muscle cell ceases. Cells switch to anaerobic glycolysis as the primary source of energy production. As a result, lactic acid accumulates, leading to a drop in the pH within the myofibre. The ultimate pH attained in postmortem muscle tissue is usually about 5.6. This pH is reached between 8 and 24 hours in beef animals, depending on antemortem conditions, carcass size and method of cooling. Although this degree of acidity may inhibit some of the enzymes in muscle cells, the majority remain active (Scopes, 1974). Rapid fall in pH, to below 5.4 in muscle tissue from hyperexcited pigs may produce meat that is pale, soft and exudative (PSE). Alternatively, in beef muscle with low glycogen reserves, pH fall may be slow and slight resulting in muscle tissue with a pH of 6.0 or above. This results in meat that is dark, firm and dry (DFD) (Briskey et al., 1966). Both of these conditions in meat lead to rejection by consumers and a lower price paid to producers.

ATP is the energy source used by the muscle cell to carry out vital functions such as maintenance of ionic gradients and the transfer of materials in and out of the cell. In the absence of Ca^{++}, ATP remains bound to the myosin head as the thick and thin filaments slide past one another. A major drain of the energy reserves in postmortem muscle cells is the breakdown of ATP by myosin ATPase. Although the rate of ATP hydrolysis is much less than in contracting muscle, this loss represents a significant load on the vital energy supply since, in the absence of oxygen, ATP regeneration cannot take place efficiently. As time passes, the supply of ATP is used up and, by the time the muscle

tissue has reached a pH of 5.6, 90% of the ATP has been depleted (Bendall, 1973). As the supply of ATP falls, the calcium ion pumps can no longer operate to capacity. Coupled with this is the fact that the membrane permeability of the sarcoplasmic reticulum increases as the pH drops. These two factors lead to a net influx of calcium ions which eventually accumulates intracellularly to a level which permits muscle contraction. This phenomenon occurs in living muscle when the Ca^{++} concentration increases from 10^{-8}M in resting muscle to 10^{-6} or 10^{-5}M in contracting muscle. However, unlike living myofibres, in which the concentration of ATP is ~6 μmol/g of muscle, the ATP concentration in postmortem muscle tissue becomes so low that the actin and myosin cross-bridges cannot be broken after the cycle. At this point, the muscle tissue is said to be in a state of rigour mortis, which literally means "stiff death". In this condition the meat is very tough and inextensible (Dransfield and Rhodes, 1975). During the onset of rigour mortis, the unrestrained muscle may contract to as little as 50% of the length of that found in the relaxed state (Bendall, 1973).

Meat reaches a maximum toughness at about 24 hr postmortem, depending on the muscle and the storage conditions (Dransfield and Rhodes, 1975). Upon further ageing, toughness decreases in the muscle tissue, reaching a minimum after 7 to 10 days. Defining these changes during ageing, and understanding the mechanisms underlying these changes is very important to the meat industry.

Breakdown of Stromal Proteins During Meat Ageing

Stromal proteins form the connective tissue and associated fibrous proteins in muscle tissue. Although stromal proteins contribute to overall meat toughness, little evidence has been produced to indicate that they are hydrolyzed during the conditioning period (Pierson and Fox, 1976). High temperatures, coupled with prolonged heating times, are required to bring about breakdown of stromal proteins in muscle tissue. Lawrie (1966) for example, demonstrated that complete gelatinization of collagen occurs during prolonged periods at temperatures between 70°C and 100°C. He also demonstrated that the increase in meat tenderness during cooking is due largely to breakdown of collagen.

Breakdown of Cytoskeletal Proteins During Meat Ageing

The majority of postmortem tenderization in muscle tissue seems to be due to changes in myofibrillar and cytoskeletal proteins (Penny, 1980). As mentioned earlier, cytoskeletal proteins are thought to play a role in maintaining the integrity of myofibres. It follows that their breakdown during the ageing period could contribute to the tenderization of meat. Young et al. (1980) showed by SDS-PAGE that desmin progressively disappears during the conditioning of meat. These researchers presumed that desmin was hydrolyzed by endogenous proteinases during the ageing period. Connectin is a cytoskeletal protein that is thought to be a major structural component of the gap or G filaments (Locker and Leet, 1976). These researchers proposed that the gap filaments are present in the core of thick filaments and extend

through the Z line connecting adjacent sarcomeres. It is thought that the gap filaments contribute to some of the elastic properties of skeletal muscle. Davey and Graafhuis (1976) demonstrated that these G filaments disappear during the ageing of meat. It was later discovered by Dayton et al. (1981) that the G filaments are susceptible to hydrolysis by CANP. Nelson and Traub (1982) revealed that the cytoskeletal proteins desmin and filamin, which are present in the Z line, could be hydrolyzed by CANP in vivo. Although these observations are only circumstantially related, the disappearance of these proteins may be a factor in the resolution of rigour mortis.

Breakdown of Myofibrillar Proteins During Meat Ageing

Although the bulk of muscle tissue is made up of thick and thin filaments, research has revealed little evidence that these components of the myofibril break down during the ageing process. With the aid of the electron microscope and SDS-PAGE, the thick and thin filaments and their constituents were shown to remain virtually unchanged during a storage period of 13 days at 2°C and 16°C (Goll and Robson, 1967; Stromer et al., 1967b). As well, there was no evident disruption of the actomyosin complex during conditioning, which implies that the thick and thin filament interactions have little effect on postmortem tenderization of meat. In vitro studies by Penny (1980) revealed that limited hydrolysis of tropomyosin, actin, myosin, C and M proteins and troponins C and I could be achieved using endogenous muscle proteinases. His work showed that troponin T was the only identifiable contractile protein degraded to any extent. The evidence

so far indicates that some other factors must be involved in the resolution of rigour mortis.

Breakdown of the Z Line During Meat Ageing

Both prerigour and postrigour muscles are extensible under a stretch load, but unlike prerigour muscle, postrigour muscles are inelastic. Thick and thin filaments slide past each other under load in prerigour muscle, whereas the extensibility of postrigour muscle is due to fracturing of the Z discs (Davey and Dickson, 1970). It seems logical, therefore, that one major factor leading to the resolution of rigour mortis is the breakdown of structural proteins in the area of the Z line. Proof of this was given by Culler et al. (1978) when they demonstrated that an increase in the amount of fracturing of the Z line corresponds to an increase in tenderness of meat over a period of 10 days. Since it is known that alpha-actinin is one of the major constituents of the Z line, it has been suggested that the breakdown of this protein could result in a loss of integrity of the Z line. However, Chin-Sheng and Parrish (1978) found that alpha-actinin is released intact from the Z disc during muscle ageing. Since alpha-actinin is not hydrolyzed in this process, the breakdown of other structural constituents must be responsible for the release of alpha-actinin during the resolution of rigour mortis (Penny, 1980). Nagainis and Wolfe (1982), demonstrated the presence of an actin-like protein in the Z line of myofibrils and suggested that hydrolysis of this protein might explain some of the weakening of the Z disc during conditioning. In fact, they found that Z disc actin was extremely

sensitive to the endogenous proteolytic enzyme CANP. If Z disc actin is a major structural component of the Z line, its hydrolysis during ageing could be a major contributing factor to postmortem tenderization of meat (Goll et al., 1983a; Nagainis et al., 1983).

PROTEOLYTIC ENZYMES AND THEIR EFFECT UPON THE RESOLUTION OF RIGOUR MORTIS

The extent of the role that proteolytic enzymes play in the resolution of rigour mortis is still not well understood. As early as 1917, Hoagland suggested that postmortem tenderization of muscle tissue is achieved by a combination of physical and proteolytic agents. Proteinases are believed to be the main factor resulting in the loss of myofibrillar and cytoskeletal integrity.

There are several classes of proteolytic enzymes that have been isolated from muscle tissue. The names, locations and catalytic classes of the main proteolytic enzymes which may lead to the breakdown of muscle proteins are listed in Table 1.

Table 1 : Proteinases in muscle tissue (Goll et al., 1983a).

Nonlysosomal

- Alkaline proteinase (serine)
- ATP dependent proteinase (unknown)
- Neutral Proteinase (cysteine?)
- High MW cytosolic proteinase (cysteine?)
- Calcium activated neutral proteinases (cysteine)

Lysosomal

Cathepsin A (serine)
Cathepsin B_1 (cysteine)
Cathepsin B_2 (cysteine)
Cathepsin C (cysteine)
Cathepsin D (carboxyl)
Cathepsin G (cysteine)
Cathepsin L (cysteine)

Alkaline Proteinase

An alkaline serine proteinase has been isolated from muscle tissue, but not specifically from muscle cells. Immunological studies have shown that this enzyme is present in granules of mast cells that are located in muscle fibres (Kay, 1984). Drabikowski et al. (1977) observed a loss of proteolytic activity in muscle tissue following an injection of the compound "48/60". This compound is a product of condensation of N-methyl-homoanisylamine with formalin and disrupts mast cells in the muscle tissue and inhibits the alkaline serine proteinase. The mast cells are known to contain serine proteinases or chymases, active in the neutral to alkaline pH range. Although chymase has the ability to degrade myofibrillar proteins in vitro, it is unlikely that this enzyme hydrolyzes muscle proteins in vivo unless there is a postmortem release of mast cell granular contents.

ATP Dependent Proteinase

Recent evidence brought forth by Etlinger et al. (1984) suggests that ATP dependent proteolysis plays a role in protein degradation in living muscle cells. They discovered that inhibition of protein breakdown in reticulocytes occurs when the ATP level drops to 20-30% of the

initial concentration. The action of ATP appears controversial in this system. Ciechanover et al. (1978) propose that the function of ATP *in vivo* is to link the polypeptide ubiquitin to target proteins enabling the proteinases to recognize these compounds as targets for degradation. Goldberg and Boches (1982) suggest that ATP acts directly on proteinases to stimulate catabolism of proteins. ATP dependent proteolysis may be partly responsible for muscle breakdown *in vivo*. To what extent this proteolytic system operates postmortem has yet to be determined.

Neutral Proteinase

Kar (1982) isolated proteinases in concentrations higher than normal from muscle samples obtained from patients with Duchenne muscular dystrophy. He discovered a neutral thiol proteinase which could affect the rate and extent of atrophy in muscle tissue. This enzyme is able to break down low molecular weight peptides into amino acids *in vitro*. Until more work is undertaken in this area, the role of this proteinase in muscle cells postmortem can only be conjectured.

High Molecular Weight Cytosolic Proteinase

Dahlmann et al. (1985) isolated a high molecular weight protein from postmortem rat skeletal tissue that contained several multicatalytic proteolytic enzymes. These enzymes exhibit pH optima of 7.5 to 10.5, depending on the substrate. The molecular weight of this enzyme is 650,000 Daltons, and the active site(s) is believed to be occupied by a cysteine residue. Although these enzymes have been shown to

degrade insulin and glucagon in vitro, as yet, research elucidating the role this enzyme plays in protein breakdown in postmortem muscle tissue has not been undertaken.

Calcium Activated Neutral Proteinase

Calcium activated neutral proteinase is a nonlysosomal thiol endopeptidase which has been isolated from muscle cells (Dayton et al., 1976). This enzyme is believed to function in protein catabolism in postmortem muscle tissue. The method of activation of this enzyme is not known, but it is thought that calcium ions bind to the enzyme, causing a conformational change. Kazutomo et al. (1983), suggest that calcium ions open up the active site of the enzyme, allowing its thiol group to catalyze the breakdown of the protein substrate.

Two isoforms of CANP have been isolated from several cell types, but only in two distinct forms (Murachi et al., 1981; Goll et al., 1983b). One form exhibits optimal activity at a Ca^{++} concentration of 30μM while the second form does not become active until the Ca^{++} concentration reaches 0.3 to 0.5mM. These proteinases exist as dimers of 80,000 and 30,000 Daltons, and are both inactivated by inhibitors such as leupeptin, EDTA and iodoacetic acid. Researchers have speculated that the mM form of CANP may be a storage form for the μM CANP (Goll et al., 1983a). They based this assumption on the fact that autolyzed mM CANP requires a lower Ca^{++} concentration to become active than before autolysis. These researchers suggested that a small peptide is cleaved from the 80,000 Dalton subunit of mM CANP which dramatically reduces the Ca^{++} concentration required for optimum activity. Dayton

et al. (1981b) demonstrated that antibodies specific for one form would cross react with the other form. They suggested that the difference between the two forms may be due to posttranslational modification. More recently, however, Penny et al. (1985) showed that antibodies specific for one form would not cross react with the other form unless the enzymes were denatured. The researchers stated that the difference was probably due to dissimilar amino acid composition resulting in different conformations in the natural states.

Some evidence is available to suggest that CANP plays a limited role in postmortem protein degradation in myofibres. Dayton et al. (1976) and Nelson and Traub (1982) demonstrated that several intracellular proteins in muscle cells are susceptible to cleavage by CANP. These researchers found that troponin I, C and T, desmin, filamin, C and M protein and tropomyosin can be degraded by this enzyme. Incubation of a solution of myofibrils with CANP released intact myosin, actin and alpha-actinin. Penny et al. (1974) demonstrated that if CANP was added to muscle tissue, it became significantly more tender after only 2 days of ageing. Although CANP acts mainly on large proteins, recent evidence by Mantle et al. (1985) indicates that limited hydrolysis of smaller peptides may result from the action of this enzyme. Immunohistochemical studies have shown that CANP is located near the Z line of myofibrils (Dayton et al., 1981a). Since CANP is known to attack the Z line of myofibrils, releasing intact alpha-actinin, it may have a role in the disassembly of myofibrillar proteins, both <u>in vivo</u> and during postmortem ageing.

Lysosomal Proteinases

The last group of proteinases believed to play a major role in meat tenderization are the lysosomal enzymes known as cathepsins. Lysosomal proteinases are a diverse group of enzymes which have been isolated from a number of cell types, including myofibres. Immunocytochemical observations have shown that Cathepsins A, B_1, B_2, C, D, H, and L are present in skeletal muscle (Bird et al., 1981). They have molecular weights ranging from 20,000 to 100,000 Daltons and exhibit pH optima from 2.0 to 8.0. Poole and his co-workers (1978) revealed that the pH of lysosomes in peritoneal macrophages is 4.5 with the aid of a fluorescein-labelled dextran. The macrophages endocytose the dextran and, in the fluorescence microscope, the substance appeared localized in granules thought to be lysosomes. This compound emits a pH dependent excitation spectra which these researchers found to correspond to a pH of 4.5. Dutson (1983) suggested this is the optimal pH for most lysosomal proteinases. The majority of cathepsins exhibit maximum activity at or below pH 5.0 and show little activity at neutral pH, although Cathepsin H is optimally active at pH 7.0. The bulk of cathepsins are thiol carboxy endopeptides, but serine enzymes have been isolated which have the ability to cleave both C terminal and N terminal residues from peptides (Mason et al., 1984).

The role of lysosomal proteinases in the conversion of muscle to meat has been controversial ever since these enzymes were first isolated from skeletal muscle. Lysosomal enzymes which are able to degrade cytoskeletal proteins and myofibrillar proteins such as actin, myosin, alpha-actinin, troponin T and troponin I have been isolated in

myofibres (Hirao et al., 1984). The majority of lysosomal proteinases have pH optima below 5.5 to 6.0, which is the ultimate pH of postmortem muscle tissue, although some activity is retained in this range. A good example of this is Cathepsin L, isolated by Kirschke et al. (1982). This enzyme has the ability to hydrolyze actin, myosin, alpha-actinin, troponin T and I and collagen into lower molecular weight peptides. Cathepsin L is optimally active at pH 4.8, but retains the ability to degrade muscle proteins at pH 6.5 at a reduced rate.

Dutson (1983) demonstrated that the high initial temperature of postmortem muscle tissue resulted in the disruption of lysosomal membranes. Postmortem pH fall to below 6.0, also disrupts lysosomal membranes, releasing cathepsins into the cytosol. The combination of lower pH and high temperature created a favourable environment for the action of cathepsins on structural and contractile proteins in muscle tissue. As would be expected, when this condition arose, meat tenderness increased substantially (Dutson, 1983). Conversely, Dransfield et al. (1981) found that higher postmortem pH, above 6.0, resulted in meat that was more tender than meat from tissue having normal postmortem pH. This situation would favour the action of nonlysosomal proteinases over cathepsins.

Researchers can only speculate on the role that each proteinase plays in the conditioning of meat. Evidence brought forth thus far suggests that the postmortem tenderization of meat is probably due to the activity of a combination of the endogenous proteolytic enzymes found in muscle tissue.

SIMILARITIES AND DIFFERENCES BETWEEN Z DISC ACTIN AND THIN FILAMENT ACTIN

Since the Z line seems to be the component of the myofibril that weakens most during ageing, it seems logical to investigate the structural proteins responsible for the integrity of the Z line. As mentioned earlier, Gard and Lazarides (1979) discovered an actin-like protein in the Z disc which was insoluble in 0.6M KI. More recently, Nagainis and Wolfe (1982) found that an actin-like protein, isolated from the Z line, is susceptible to hydrolysis by CANP. In addition to the difference in solubility of Z disc actin and thin filament actin in 0.6M KI, these researchers showed that the two actins have slightly different isoelectric points. Using reverse phase high performance liquid chromatography, Chang et al. (1985) revealed that the profiles of the cyanogen bromide cleaved peptides differed slightly between the Z line actin and the thin filament actin. Nagainis and Wolfe (1982) produced antibodies to the KI insoluble actin and used immunofluorescent microscopy to determine that the actin-like protein resided primarily in the Z line. Subsequent incubation of these myofibrils with CANP revealed that both the Z lines and fluorescence were removed. This experiment not only suggested that Z actin is present in the Z line, but also proposed that this protein is susceptible to hydrolysis by CANP. However, their work also revealed similarities between the two actins. Antibodies produced against Z disc actin exhibited analogous immune responses towards both actins *in vitro*. Amino acid composition of the two actins was virtually identical and the two proteins were shown to have molecular weights of 42,000 Daltons, as illustrated by

using SDS-PAGE. Whether the KI insoluble actin present in the Z line of myofibrils is distinct from thin filament actin has not yet been proven. More work has to be undertaken before this Z disc actin can be fully characterized. Until this is done, one can only speculate as to the function of this protein in the myofibril and its effect upon the tenderization of meat.

Our knowledge of the complex series of events causing the resolution of rigour mortis is more complete than a few short years ago. We now have a greater understanding of the function of many of the structural proteins in the myofibre. Proteolytic enzymes have been discovered which are thought to participate in the breakdown of muscle tissue. However, there still exist a myriad of unexplained occurrences which bring about the tenderization of meat.

STATEMENT OF THE PROBLEM

Several mechanisms have been postulated to explain the resolution of rigour mortis. These mechanisms range from the breakdown of certain cytoskeletal proteins in myofibres, (Davey and Graafhuis, 1976), to the hydrolysis of structural proteins in the Z line (Penny, 1980; Chin-Sheng and Parrish, 1978). The proposal that an actin-like protein exists in the Z line and is susceptible to CANP is interesting in that it may partially explain the fragmentation of myofibres during post-mortem conditioning which apparently results in the tenderization of meat. It is not yet known whether Z disc actin is completely distinct from thin filament actin or, whether the different properties of the proteins are due to partial denaturation or posttranslational modifica-

tion. The difference between Z disc actin and thin filament actin could ideally be determined by analytical techniques such as X-ray crystallography and amino acid sequencing. However, due to limitations in equipment and expertise in these fields, other methods were used to analyze Z disc actin and thin filament actin. The research presented in this thesis is an attempt to characterize Z disc actin and to determine if fundamental differences exist between it and thin filament actin. If such differences exist, and these contribute to the properties which make the Z disc different from the thin filament, one of the mysteries regarding the postmortem tenderization of muscle tissue would be solved.

MATERIALS AND METHODS

All protein purification steps were carried out at 4°C unless otherwise stated. To prevent the growth of microorganisms, 0.01% (w/v) sodium azide (NaN_3) was added to all buffers. Unless otherwise specified, chemicals used were of reagent grade obtained from Sigma Chemical Company, Aldrich Chemical Company Ltd., or Mandel Scientific Company. See appendix 2 for more information on the chemical compositions of the buffers used in the experimental procedures. All pH determinations were carried out at 25°C, before the addition of reducing compounds such as ß-mercaptoethanol (ß-MCE) or dithiothreitol (DTT). Dialysis tubing and cheesecloth were boiled in distilled water and 0.01% (w/v) NaN_3 and stored at 4°C prior to use. The cheesecloth and dialysis tubing were stored not longer than 2 weeks before use. The dialysis tubing had a molecular weight (MW) cutoff of 12,000-14,000 Daltons (Fisher Scientific Company). The preparative centrifuge used was a Beckman J2-21, while high speed centrifugation was performed on a Beckman L2-65B ultracentrifuge. All g forces given are average values.

Protein Determination

Protein quantitation was by the dye binding procedure developed by Bradford (1976) and modified by Read and Northcote (1981). The dye used was Coomassie Blue G (Serva) and the protein-dye complex absorption was measured in a Bausch and Lomb Spectronic 21 spectrophotometer at 595 nm. Protein concentration was obtained from a standard curve using

bovine serum albumin (BSA) prepared from lyophilized powder (Sigma).

Protein Concentrating

Whenever necessary, protein solutions were concentrated using an Amicon Model 8200 ultrafiltration cell under an atmosphere of nitrogen at 240 Kpa. PM 10 diaflo ultrafilters were used which had a MW cutoff of 10,000 Daltons.

Polyacrylamide Gel Electrophoresis

Purity of all protein preparations was monitored using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). See appendix 1 for the preparation of gels, MW standards and protein samples for SDS-PAGE. The procedure used was a modification of the slab gel method outlined by Laemmli (1970). All gels were run on either the Mighty Small Slab Electrophoresis Unit, SE 200 (Hoefer Scientific Instruments) or the Protean II electrophoresis unit (Bio-Rad Industries).

Muscle Tissue Preparation

Chicken pectoral muscle was excised immediately after exsanguination from 6 to 10 week old broilers (Lilydale Poultry Farms, Edmonton, Alberta). The muscle tissue was rinsed with distilled water and all visible connective tissue was removed. The muscle tissue was cooled to 4°C and minced for 10 sec in a Moulinex food processor. This procedure was repeated for all protein isolations.

Z DISC ACTIN PURIFICATION

Myofibril Preparation

Myofibrils were prepared from the minced muscle using a modification of the procedure described by Hay et al. (1973). The muscle tissue was placed in 10 volumes of a myofibril buffer (appendix 2), homogenized in a Waring blender for 3 sec and spun at 1,000 x g for 10 min in a Beckman JA-14 rotor. The supernatant was discarded and the sediment was suspended in 6 volumes of myofibril buffer. This solution was spun at 1,000 x g for 10 min. The sediment was homogenized for 3 sec in a Waring Blender in 8 volumes of myofibril buffer and passed through a household net strainer to remove residual connective tissue. The suspension was centrifuged as described previously. This straining and homogenization step was repeated and the sediment was suspended in 6 volumes of myofibril buffer plus 1% (v/v) Triton X-100 (BDH) and centrifuged at 1500 x g for 10 min (all subsequent centrifugations were carried out at 1500 x g). This step was repeated and the sediment was placed in 8 volumes of myofibril buffer, stirred vigourously with a polyethylene rod for 10 min and centrifuged. This step was repeated 5 times with 0.1M KCl substituted for the myofibril buffer. After the final centrifugation, the sediment was suspended in 2 volumes of 0.1M KCl by homogenizing for 2 sec in a Waring blender. The protein concentration of the myofibril solution was usually between 10 and 15 mg/ml. This suspension was stored at 4°C until required.

Extraction of Z Disc Actin from Myofibrils

Ninety ml of myofibril suspension (15 mg/ml) were placed in a JA-20 rotor and centrifuged at 1,500 x g for 10 min. The sediment was quickly suspended in 1 1/2 volumes of buffer Z (appendix 2) and centrifuged for 2 min at 3,500 x g in the JA-14 rotor. This step was repeated twice in low ionic strength buffer (LISB) (appendix 2). Since it is known that extraction of myofibrils with LISB could remove the proteins in the Z line, (Stromer et al., 1967a), this method was used to begin the isolation of Z actin from chicken pectoralis muscle. The sediment from the last centrifugation was placed in 2 1/2 volumes of LISB and gently stirred with a polyethylene rod for 24 hr. The solution of myofibrils was centrifuged at 30,000 x g for 2 1/2 hr in the JA-20 rotor to pellet the insoluble components. The supernatant, containing mainly Z actin and some other contaminating myofibrillar proteins, was saved while the sediment was discarded.

Further purification of Z actin was accomplished using a modification of the procedure outlined by Pardee and Spudich (1982) for isolation of thin filament actin. Solid KCl, $MgCl_2$ and ATP was added to the supernatant to bring the concentrations to 100mM, 2mM and 1mM, respectively. The solution was allowed to stand for 2 hr at 25°C while polymerization of the Z actin monomers took place. Solid KCl was added to the solution to bring the concentration to 0.6M. The mixture was stirred for 1/2 hr to solubilize contaminating proteins. The solution was placed in a Beckman Ti-50.2 rotor and centrifuged for 2 hr at 160,000 x g. Incubation with CANP and subsequent SDS-PAG electrophoretic analysis was used to determine the presence of Z disc

actin. The Z actin pellets were solubilized in 20 ml of buffer Z-1 (appendix 2) and stored at 4°C until needed. Plate 2 is a photograph of an SDS-polyacrylamide gel showing the various steps involved in the purification of Z actin. The high MW contaminating proteins are thought to originate from the M line since these proteins have molecular weights ranging from 145,000 to 185,000 Daltons. The M line proteins are closely associated with the thick filaments and evidence suggests that they do not interact with actin. Therefore, their presence in the Z actin solutions was thought not to have a pronounced effect on the outcome of the various experiments. It was for this reason that further purification of the Z actin solutions was not undertaken.

THIN FILAMENT ACTIN PURIFICATION

Muscle Acetone Powder Preparation

Muscle acetone powder (MAP) was prepared based on the method described by Pardee and Spudich (1982). Three hundred and fifty grams of muscle tissue were rinsed with 0.2M ethylenediaminotetraacetic acid (EDTA) and minced as described previously. The muscle mince was extracted in 1 litre of KCl buffer (appendix 2) for 10 min with a polyethylene stirring rod. The solution was then passed through a double layer of cheesecloth. This step was repeated after every wash. The muscle mince was placed in 2 l of 0.05M sodium bicarbonate ($NaHCO_3$), pH 8.0 and stirred for 10 min After passing through the cheesecloth, the muscle mince was stirred in 1 l of 1mM EDTA for 10 min. The next 2 steps in the muscle acetone powder preparation involved extractions

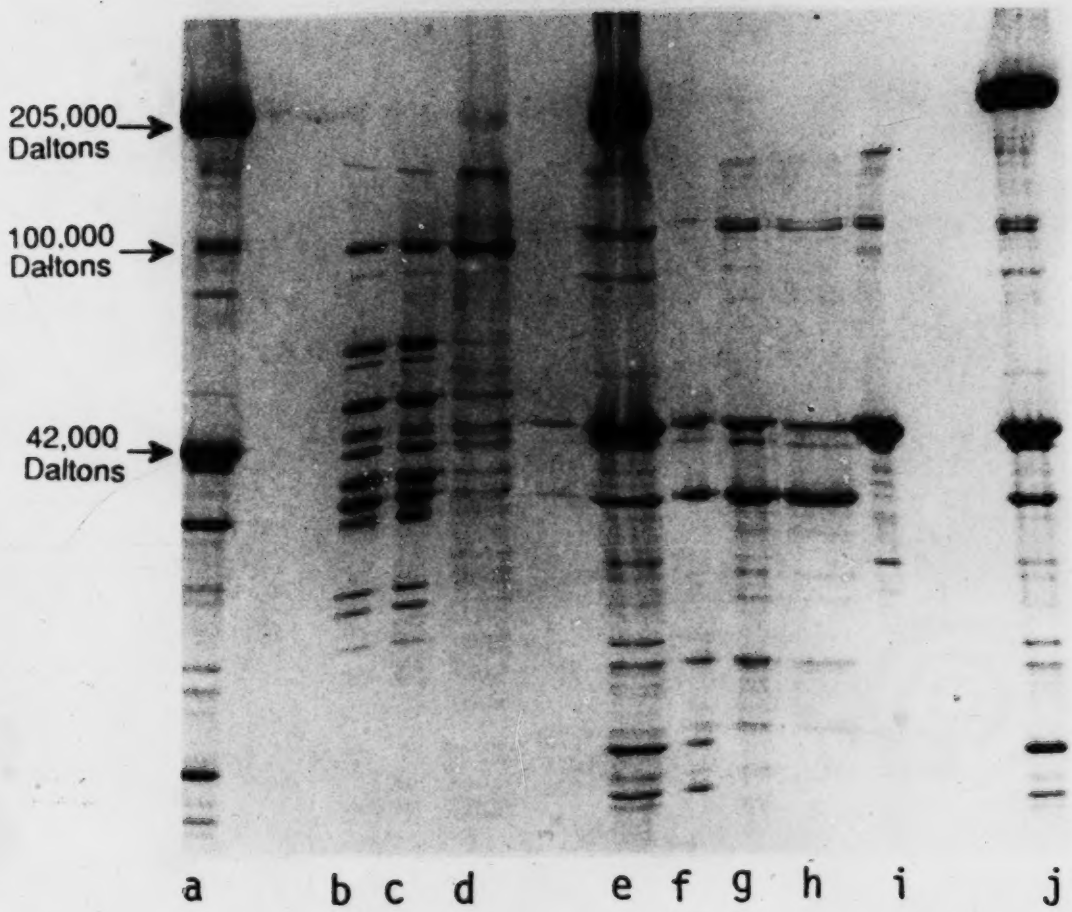

Plate 2. SDS-PAGE illustrating the steps involved in the preparation of myofibrils and isolation of Z actin. (a) myofibril solution (used as a MW standard); (b) supernatant from myofibril buffer extraction (appendix 2); (c) supernatant from Triton X-100 and myofibril buffer extraction; (d) supernatant from 1st 0.1M KCl wash; (e) purified myofibrils; (f) solution after 24 hr extraction in LISB (appendix 2); (g) supernatant after centrifugation at 30,000xg; (h) supernatant after 0.6M KCl wash and centrifugation at 160,000xg; (i) Z actin pellet; (j) standard myofibril solution.

in 2 l of distilled water for 5 min each. The next five washes were performed at room temperature using 1 l of acetone for each extraction. The mince was stirred for 10 min in each step and, after the last filtration, the acetone mince was allowed to dry overnight on a large evaporating dish. Typical yields of MAP were about 10-15 g per 350 g of muscle tissue. The MAP was stored at -20°C until needed. Plate 3 is a photograph of an SDS-polyacrylamide gel of the various extraction steps involved in the preparation of MAP.

Extraction of Thin Filament Actin from MAP

F actin was purified from muscle acetone powder as described by Pardee and Spudich (1982). Ten g of MAP typically yielded 100-300 mg of F actin. The MAP was placed in 200 ml of actin buffer (appendix 2) and stirred by hand with a stainless steel spatula for 30 min. The solution was centrifuged for 15 min at 7,500 x g in a JA-14 rotor. The supernatant was passed through 4 layers of cheesecloth and saved while the residue was re-extracted in 200 ml of actin buffer for 10 min. The second extract was passed through the cheesecloth and the combined filtrates were centrifuged for 1 hr at 10,500 x g. The supernatant was saved and solid KCl, $MgCl_2$ and ATP were added to bring the concentration to 0.1M, 2mM and 1mM respectively. The globular (G) actin monomers were allowed to polymerize for 2 hr at 25°C to form filamentous (F) actin. Solid KCl was added to the solution to bring the concentration to 0.6M. The actin suspension was stirred for 1/2 hr at 25°C to solubilize contaminating proteins. The solution was placed in a Beckman Ti-50.2 rotor and centrifuged for 2 hr at 160,000

x g. The actin pellets were washed twice and suspended in 20 ml of actin-1 buffer (appendix 2) with a glass-teflon ball homogenizer. The actin suspension was stored at 4°C until needed. Plate 3 is a photograph of an SDS-polyacrylamide gel which includes of the various steps in the purification of F actin from MAP.

MYOSIN PURIFICATION

The myosin purification procedure used was a modification of the method outlined by Margossian and Lowey (1982). Four hundred grams of muscle mince were prepared as mentioned previously. The myofibrils were extracted in 2 volumes of buffer A (appendix 2) with an overhead paddle-type stirrer for 15 min. Four volumes of distilled water were added to the solution and the mixture was strained through 3 layers of cheesecloth. The filtrate was further diluted with 3 volumes of distilled water and allowed to settle for 3 hr. The supernatant was siphoned off and the sediment was spun at 5,500 x g for 15 min. The precipitate was dialyzed overnight against 3 l of buffer B (appendix 2). The suspension was placed in an equal volume of distilled water and stirred for 30 min. The solution was centrifuged at 17,000 x g for 1 hr and the supernatant obtained was diluted 8 fold with distilled water and left overnight to allow the myosin to settle. The supernatant was decanted and the sediment was centrifuged at 9,000 x g for 10 min. The insoluble protein was suspended in a minimum volume of buffer C (appendix 2) and dialyzed overnight against 3, 1 l changes of buffer A (appendix 2). The solution was centrifuged for 1 hr at 28,000 x g. The supernatant was passed through a diethylaminoethyl (DEAE) Sepharose

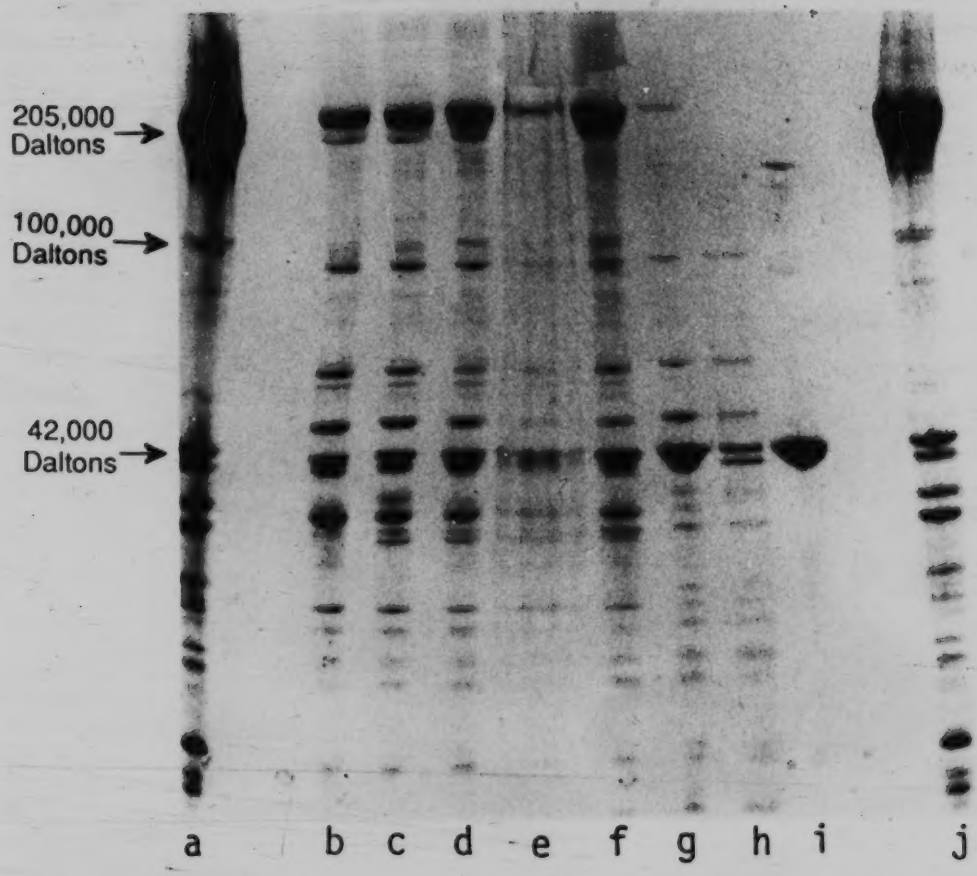

Plate 3. SDS-PAGE illustrating the steps involved in the purification of thin filament (F) actin from muscle acetone powder (MAP). (a) standard myofibril solution; filtrate from: (b) 0.1M KCl extraction; (c) 0.05M $NaHCO_3$ extraction; (d) 1mM EDTA extraction; (e) and (f) are the 1st and 2nd distilled water extractions; (g) supernatant from MAP extractions with actin buffer (appendix 2); (h) supernatant after 0.6M KCl wash and centrifugation at 160,000xg; (i) F actin pellet after polymerization and centrifugation at 160,000xg; (j) standard myofibril solution.

Fast Flow column (Pharmacia) twice using a 0.1M to 0.6M KCl gradient with the following constituents remaining the same throughout; 0.15M potassium phosphate, pH 6.5, 20mM EDTA, 5mM $MgCl_2$ and 1mM ATP. The column dimensions were 2.6 x 30 cm and the gradient volume was 2 x 1 l at a flow rate of 0.75 ml/min. The myosin peak was diluted 1:1 with glycerol and stored at -20°C until required. Plate 4 is a photograph of an SDS-polyacrylamide gel of the final steps in the purification of myosin.

ALPHA-ACTININ PURIFICATION

The procedure used for the isolation of alpha-actinin from skeletal muscle tissue was a modification of the procedure used by Arakawa et al. (1970). Three hundred grams of myofibrils were suspended in 500 ml of 0.1M KCl using a Waring blender and centrifuged at 10,000 x g for 10 min. The sediment was re-extracted by this procedure 3 more times and the combined extracts were dialyzed for 24 hr against 3, 1 l changes of alpha-actinin buffer (appendix 2). After dialysis, the suspension was placed in 2 l of the alpha-actinin buffer and extracted for 72 hr with a polyethylene stirring rod. The alpha-actinin solution was centrifuged at 30,000 x g for 30 min. Solid ammonium sulphate was dissolved in the supernatant to precipitate the proteins into 3 fractions; 0-17%, 17-30% and over 30% ammonium sulphate. The 17-30% protein precipitate was allowed to stand for 1 hr and then centrifuged at 12,000 x g for 15 min. The crude alpha-actinin sediment was suspended in 150 ml of 1mM $NaHCO_3$ and dialyzed against 3, 1 l changes of the same solvent over a 48 hr period. The solution was centrifuged at

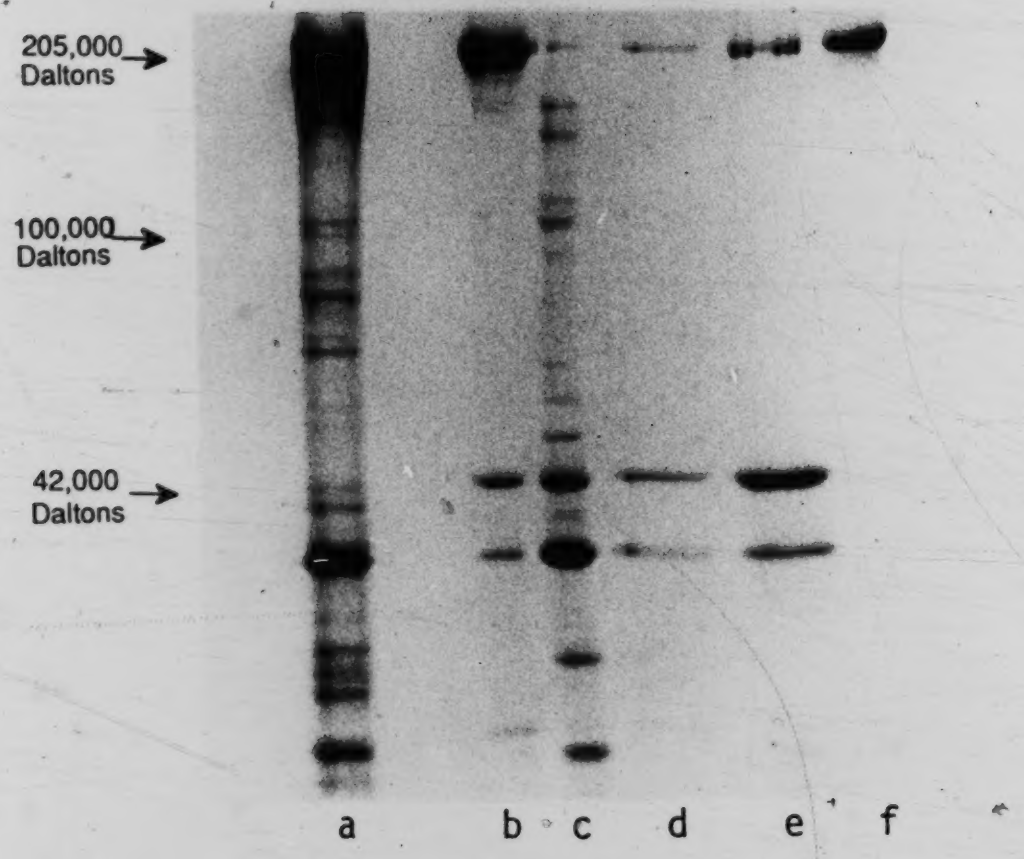

Plate 4. SDS-PAGE illustrating the steps involved in the purification of myosin. (a) standard myofibril solution. A crude myosin extract was obtained as outlined by Margossian and Lowey (1982). This extract was centrifuged at 28,000xg for 1 hour, (b) illustrates the sediment and (c) illustrates the supernatant. The supernatant was passed through a DEAE column (Sepharose Fast Flow, Pharacia) with a 0.1M KCl to a 0.6M KCl gradient yielding the following peaks; (d) peak eluting at 0.25M KCl; (e) peak eluting at 0.20M KCl; (f) peak eluting at 0.13M KCl (myosin).

50,000 x g for 1 hr and the supernatant was saved. The supernatant was applied to a 2.6 x 30 cm DEAE (Whatman DE52) column and washed with a 2 l gradient of 0.1M KCl, 10mM Tris-HCl, pH 8.2 to 0.6M KCl, 10mM Tris-HCl, pH 8.2 at a flow rate of 0.70 ml/min. Three peaks were eluted from the column and these were analyzed using SDS-PAGE. The first peak contained a mixture of proteins, the second peak contained a protein with a MW of 130,000 Daltons, while the last peak, which eluted at 0.25M KCl, had a MW of 100,000 Daltons, which corresponds to the MW of alpha-actinin. Plate 5 is a photograph of an SDS-polyacrylamide gel of the various steps in the isolation of alpha-actinin.

SCANNING ULTRAVIOLET SPECTROPHOTOMETRY

Identical concentrations of freshly purified Z actin and F actin were scanned in a Hewlett Packard 8451 A Diode Array spectrophotometer at wavelengths from 220 to 320 nm. The samples were split into 2 groups, one group was denatured by dialysis in 6M urea and dialyzed in a benign buffer (appendix 2). Dialysis was carried out over a 48 hr period with 3, 1 l changes. The other group of F actin and Z actin samples was simply dialyzed against the benign buffer. The samples were scanned in a UV spectrophotometer to determine the effect of denaturation and exhaustive dialysis on the spectra of Z actin and F actin.

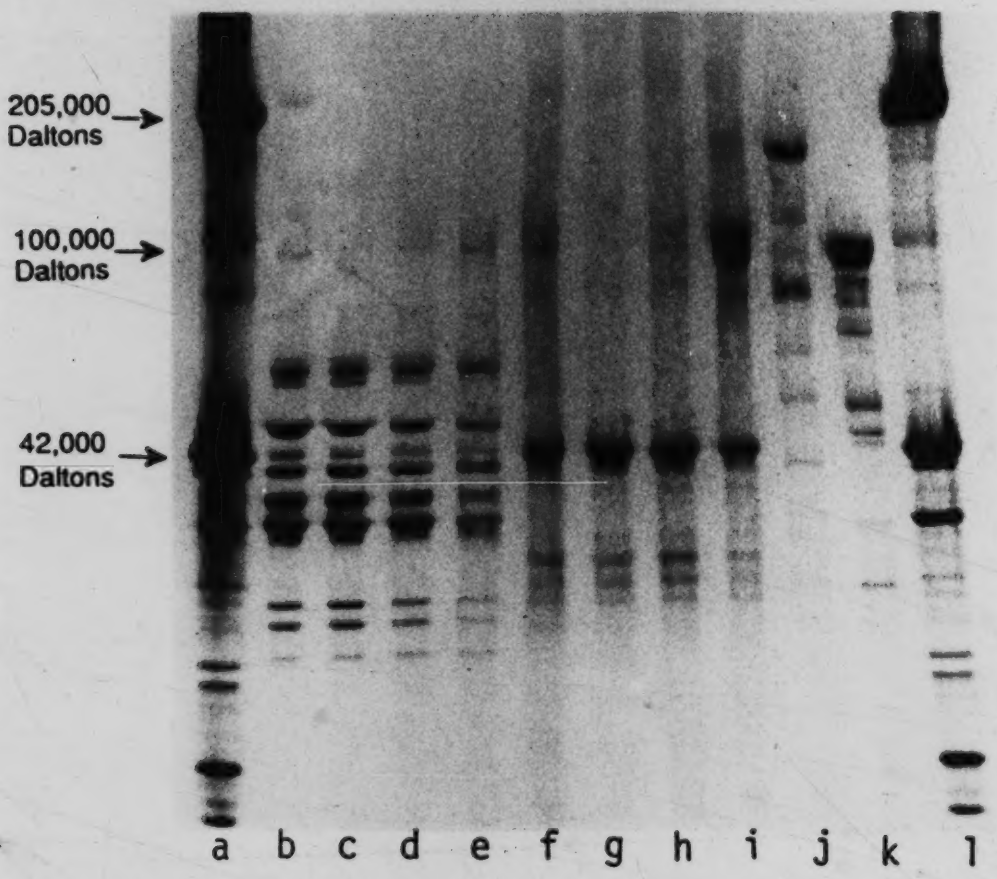

Plate 5. SDS-PAGE illustrating the steps involved in the purification of alpha-actinin. (a) standard myofibril solution; lanes (b) to (e) represent the filtrates from the 0.1M KCl extractions; (f) solution after dialysis in alpha-actinin buffer (appendix 2); (g) precipitate from 0-17% ammonium sulphate (AS) extraction; (h) precipitate from 17-30% AS extraction; (i) supernatant from 17-30% AS extraction. The 17-30% AS precipitate was solubilized and passed through a DEAE (Whatman DE52) column using a gradient from 0.1M KCl to 0.6M KCl; (j) 2nd elution peak; (k) 3rd peak from column, eluting at 0.25M KCl (alpha-actinin); (l) standard myofibril solution.

Enzymatic Digestion of Z Disc Actin and Thin Filament Actin

Enzymatic hydrolysis was carried out on samples of Z actin and F actin using chymotrypsin (Worthington). Chymotrypsin was purchased as a lyophilized powder having an activity of 58.5 units/mg of protein (See appendix 1 for more information of the activity of this enzyme). Protein concentration of chymotrypsin was 0.20 mg of enzyme per 1.0 mg of Z actin and F actin. All reactions were carried out at 25°C in 0.1M sodium phosphate buffer, pH 7.8. Prior to hydrolysis, the Z and F actin solutions were dialyzed against 3, 1 l changes of the enzyme buffer over a 48 hr period. Two sets of digestions were carried out on the Z and F actin solutions using chymotrypsin. One set of reactions was allowed to proceed for 1 hr while the second set was allowed to proceed for 22 hr. All enzyme reactions were stopped by bringing the concentration of iodoacetic acid to 5mM.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY ANALYSIS OF THE CHYMOTRYPTIC DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

Analysis of the enzymatic digestion was carried out on a Beckman Model 332 Gradient Liquid Chromatograph System. After centrifugation in an Eppendorf Centrifuge 5414 for 4 min, 50 µl of the protein solution was injected into the HPLC system. A gradient of 0.1% (v/v) trifluoroacetic acid (TFA) and distilled water to 0.1% (v/v) TFA and 50% acetonitrile/distilled water was used to separate the protein solution. The gradient was run at 0 to 50% acetonitrile over a 50 min period at a flow rate of 0.8 ml/min. All solvents were HPLC grade and the distilled water was passed through a 0.45 µm millipore filter

prior to use. In addition, all solvents were degassed daily to remove dissolved gaseous impurities. Separation of the protein hydrolysate was accomplished using a 250 x 4.6 mm, C-8 Reverse Phase column (Synchropak). Analysis of the enzyme digest was performed using an LKB/Milton Roy Spectro Monitor D spectrophotometer at wavelengths of 210 and 280 nm. The peaks were plotted on a Hewlett Packard 3390A integrator.

Denaturation of Z Disc Actin and Thin Filament Actin

Isolated Z actin and F actin were dialyzed in a 6M urea buffer (appendix 2) with 4, 1 l changes over a 48 hr period. The denatured Z actin and F actin samples, along with the undenatured Z and F actin, were dialyzed against a benign buffer (appendix 2) as mentioned previously.

AGGREGATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

2.0 ml of the dialyzed Z and F actin samples were placed in a Beckman DU spectrophotometer, updated with a Gilford 252 Modernization System attachment. The protein concentration was 0.40 mg/ml and the absorbance of the samples was monitored at 340 nm with constant stirring. 3, 30 µl aliquots of 0.33M $CaCl_2$ were added, resulting in a final concentration of $CaCl_2$ of 15mM. The absorbance was plotted using a Varian A-25 chart recorder. At this point, 4 to 5 30 µl aliquots of 0.33M ATP, adjusted to pH 8.0, were added to the protein solution until another change in absorbance was observed. This procedure was repeated for both the denatured and undenatured F and Z actin

samples.

SUPERPRECIPITATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN IN THE PRESENCE OF MYOSIN

The absorbance of the denatured and undenatured actin samples was monitored in the Beckman DU spectrophotometer, as before, with stirring at 340 nm. The protein concentration of the three solutions of F actin, Z actin and myosin was 0.40 mg/ml. 0.5 ml of myosin was placed in the cuvette and the absorbance was recorded while 1.5 ml of the actin sample was added. 5, 30 µl aliquots of 0.33M ATP, adjusted to pH 8.0, were sequentially added to each actin-myosin solution until the concentration reached 25mM. The change in absorbance was measured at 340 nm, as before.

ELECTRON MICROSCOPY

Muscle Bundle Preparation

Muscle fasiculi were removed from chicken pectoral muscle and teased into ~2mm by 40mm bundles. These bundles were tied to wooden applicator sticks with nylon string and placed in a storage buffer (appendix 2).

Sample Preparation for Electron Microscopy

The muscle bundles were rinsed in 50mM cacodylate buffer, pH 7.2 and fixed overnight in Karnovsky's solution. The fibres were washed again in cacodylate buffer and fixed in 2% (w/v) osmium tetroxide

(OsO_4) for 1 hr. The samples were washed with distilled water and dehydrated in a series of ethanol washes ranging from 50% to 98%. To remove any residual water, the muscle bundles were rinsed twice in propylene oxide. The samples were then placed in a 3:2 ratio of propylene oxide: Araldite 502 overnight. The muscle bundles were inserted into moulds and embedded in an Araldite 502 solution for 48 hr at 65°C. The moulds were cut into thin sections with a DuPont diamond knife and placed onto Metaxaform copper grids, Mesh 300 (3.05 mm diameter). The sections were stained in uranyl acetate for 15 min, washed with distilled water and stained with lead citrate for 10 min. Excess stain was rinsed off with distilled water. After drying, the sections were viewed in a Phillips EM 201 electron microscope from the Biological Sciences Department, and photographed on Eastman fine grain release positive film 5302.

SDS-PAGE ANALYSIS OF THE CANP DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

Half of the Z and F actin samples were dialyzed in the 6M urea buffer (appendix 2) for 48 hr with 4, 1 l changes of the buffer. After denaturation, these samples, plus the undenatured Z actin and F actin samples, were dialyzed against the benign buffer (appendix 2) for 48 hr with 4, 1 l changes of the buffer. Extraction times for Z disc actin and thin filament actin were varied from 1 hr to 24 hr, and the proteins were purified in the usual manner. F actin, exhaustively dialyzed in the benign buffer, and freshly purified Z actin were dialyzed in actin buffer for 24 hr to depolymerize the actin filaments.

Centrifugation at 160,000 x g for 2 hr was used to pellet any insoluble actin filaments. The actin monomers were polymerized into filaments and centrifuged as mentioned previously. Seven μg of CANP (prepared in our laboratory from bovine muscle) were added to 35 μg of protein in a buffer containing 5mM $CaCl_2$. (See appendix 3 for more information on the activity of this enzyme). The reaction was allowed to proceed for 1/2 hr at 37°C and then stopped by bringing the concentration of EDTA to 10mM. The protein digests were analyzed using SDS-PAGE.

RESULTS

Several experimental approaches were followed to determine if differences exist between the KI insoluble (Z) actin and thin filament (F) actin. Evidence suggesting that there are fundamental differences between Z actin and F actin has been published (Nagainis and Wolfe, 1982); however another interpretation of their findings is that Z actin may be merely a denatured form of thin filament actin. The experimental data presented here is an attempt to determine if biochemical differences, other than denaturation, exist between F actin and Z actin.

SCANNING ULTRAVIOLET SPECTROPHOTOMETRY

Scanning the UV spectra of a 1.0 mg/ml solution of F actin from 220 to 320 nm reveals a smooth curve with peak absorbance at 260 nm. The UV spectra of a 1.0 mg/ml Z actin solution reveals an absorption spectra which differs from F actin. There is no 260 nm peak, instead the Z actin solution yields two peaks; one at 280 nm and the other at 230 nm, probably representing the aromatic amino acids and oxidized dithiothreitol, respectively. Figure 5 illustrates the absorption spectra of freshly purified samples of Z actin and F actin.

A difference spectra between 220 and 320 nm of a 1.0 mg/ml F actin solution was obtained using a 1.0 mg/ml Z actin solution as the blank. Figure 6 illustrates the absorption spectra that was obtained. This graph reveals that the F actin solution absorbed much more strongly at 260 nm than the Z actin solution.

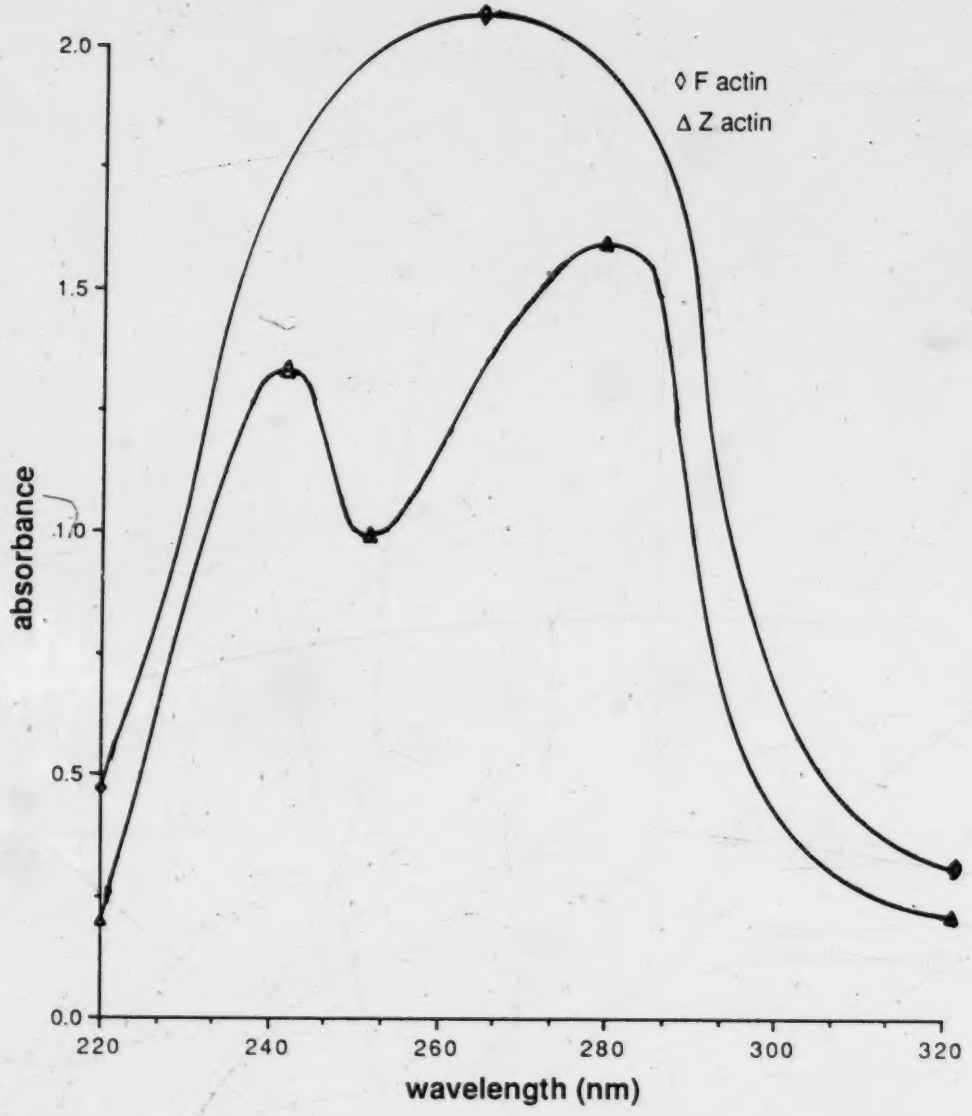

Figure 5. Scanning UV spectrophotometric analysis of freshly purified Z actin and F actin. F actin yielded one large broad peak, while Z actin produced a peak at 240 nm and another at 280 nm. Protein concentrations were 1.0 mg/ml.

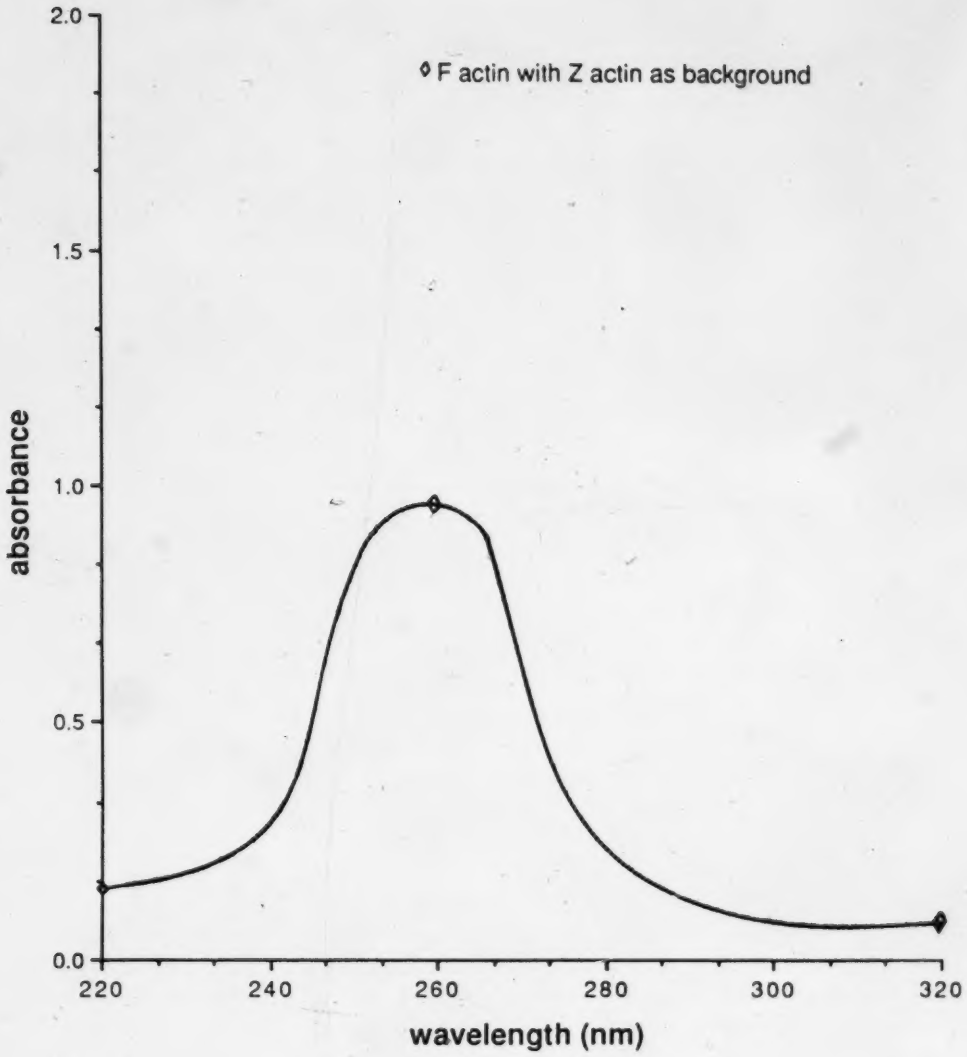

Figure 6. Scanning UV spectrophotometric analysis of F actin using Z actin as the blank. F actin yielded a large peak at 260 nm, corresponding to the absorption maxima of the adenine ring of ADP. Protein concentrations were 1.0 mg/ml.

The second part of this experiment involved denaturing half the F actin and Z actin samples in 6M urea. Both the denatured and undenatured samples were then dialyzed in a non-denaturing (benign) buffer (appendix 2) and the UV spectra were taken again. The UV spectra of the Z actin solutions remains unchanged after dialysis, however the dialyzed denatured and undenatured F actin samples reveals a loss of absorbance at 260 nm. Figures 7 and 8 are the scanning UV absorption spectra of the Z actin and F actin samples. Presumably the compound absorbing at 260 nm is not covalently bound to the F actin molecule and therefore it was lost during the dialysis. Although not shown here, the difference spectra of the denatured and undenatured F actin using the corresponding Z actin samples as blanks yielded plots that showed no appreciable absorbance at any wavelength. This evidence demonstrates that both actin samples have an overall absorption spectra after denaturation or exhaustive dialysis that are virtually identical.

HPLC ANALYSIS OF THE CHYMOTRYPTIC DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

Enzymatic hydrolysis was carried out on exhaustively dialyzed Z actin and F actin by chymotrypsin in 2 separate trials. The first digestion was allowed to proceed for 22 hr. The purpose of this experiment was to determine whether the HPLC elution profile of the peptides produced by proteolysis of Z actin were different from the HPLC elution profile of the peptides produced by F actin proteolysis. To serve as a control, HPLC elution profiles of autolysis of chymotrypsin were carried out to determine the number of peptides due to

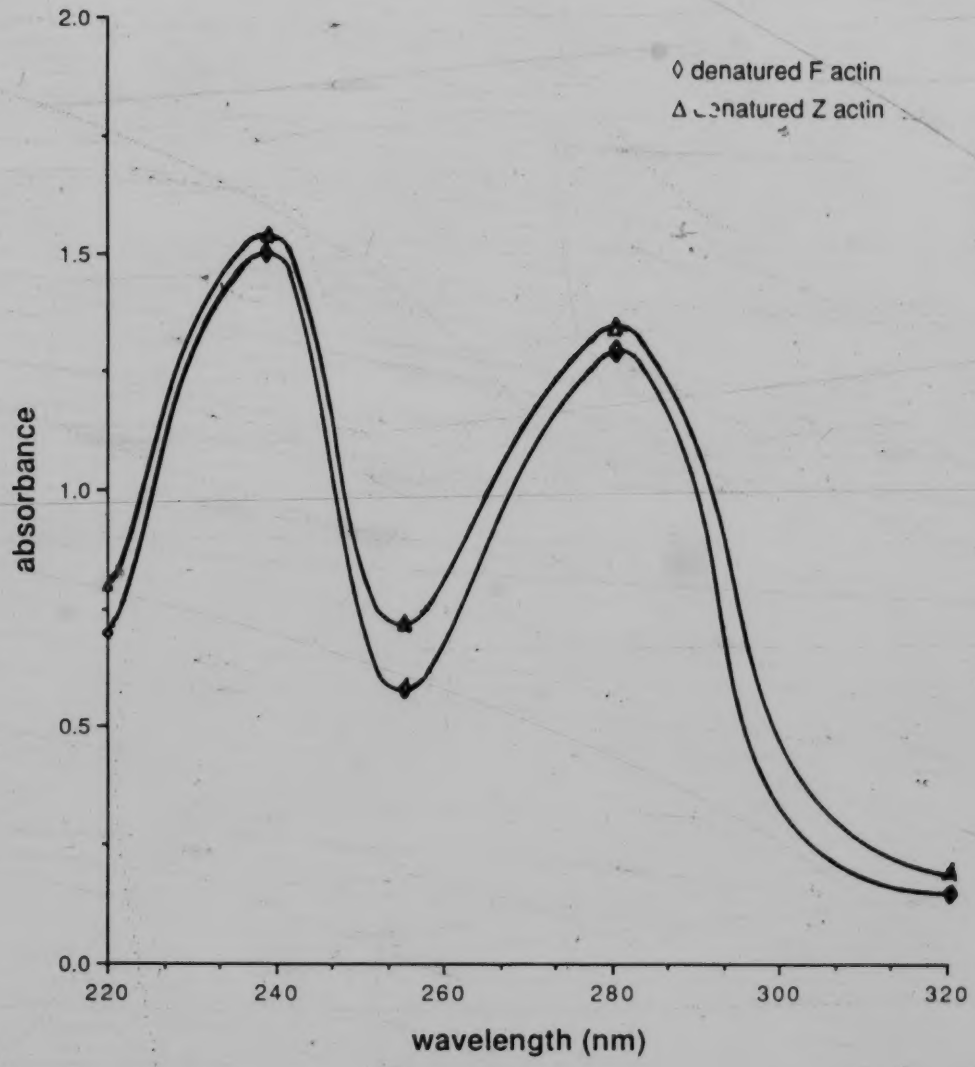

Figure 7. Scanning UV spectrophotometric analysis of F actin and Z actin denatured in 6M urea. Absorption patterns of both samples were similar at all wavelengths. The F actin sample showed a loss of the 260 nm peak as a result of denaturation. Protein concentrations were 1.0 mg/ml.

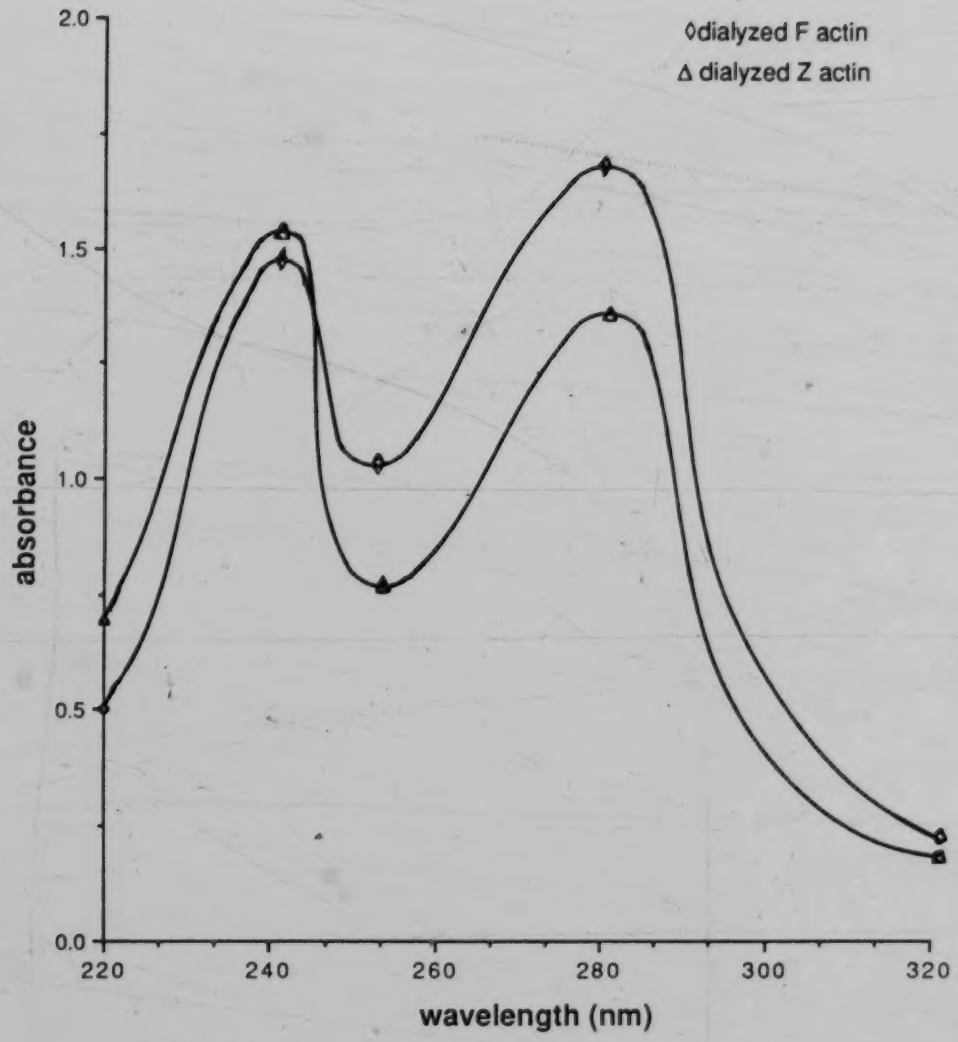

Figure 8. Scanning UV spectrophotometric analysis of F actin and Z actin exhaustively dialyzed in a benign buffer (appendix 2). Results showed a loss of the 260 nm peak in F actin. Both spectra exhibited similar absorption patterns at the wavelengths measured. Protein concentrations were 1.0 mg/ml.

the presence of the enzyme only. Although not shown here, the contribution of chymotrypsin alone to the elution profiles was insignificant compared to the peaks due to the F actin and Z actin hydrolysates. Duplicate samples of the hydrolysates were analyzed at 210 and 280 nm on a variable wavelength UV detector. Figures 9 and 10 illustrate the elution profiles of the 1 hr chymotryptic hydrolysates of Z actin and F actin. The elution profiles of F actin and Z actin samples are virtually identical at both 210 nm and 280 nm. As expected, the chromatograms of the 22 hr digestion are not the same as their 1 hr counterparts. The number of peaks increases as the hydrolysis time increases. This is probably due to the breakdown of large peptide chains into smaller peptides by the action of chymotrypsin. Figures 11 and 12 illustrate the elution profiles of the 22 hr chymotrypsin digests of Z and F actin at 210 and 280 nm. As before, the elution profiles of the Z actin and F actin hydrolysates are similar. The similarity in the elution profiles of Z actin and F actin hydrolysates indicate that peptides eluted from the column could be analogous in molecular weight and hydrophobicity.

AGGREGATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

In the presence of higher concentrations of salts such as $CaCl_2$ and $MgCl_2$, proteins will aggregate into large clumps which are easily visible with the naked eye (Lapanje, 1978). This aggregation was monitored at 340 nm in a spectrophotometer as described by White, (1982). Addition of ATP at this point caused a dissociation of the clumps leading to a decrease in absorbance and reduction in viscosity.

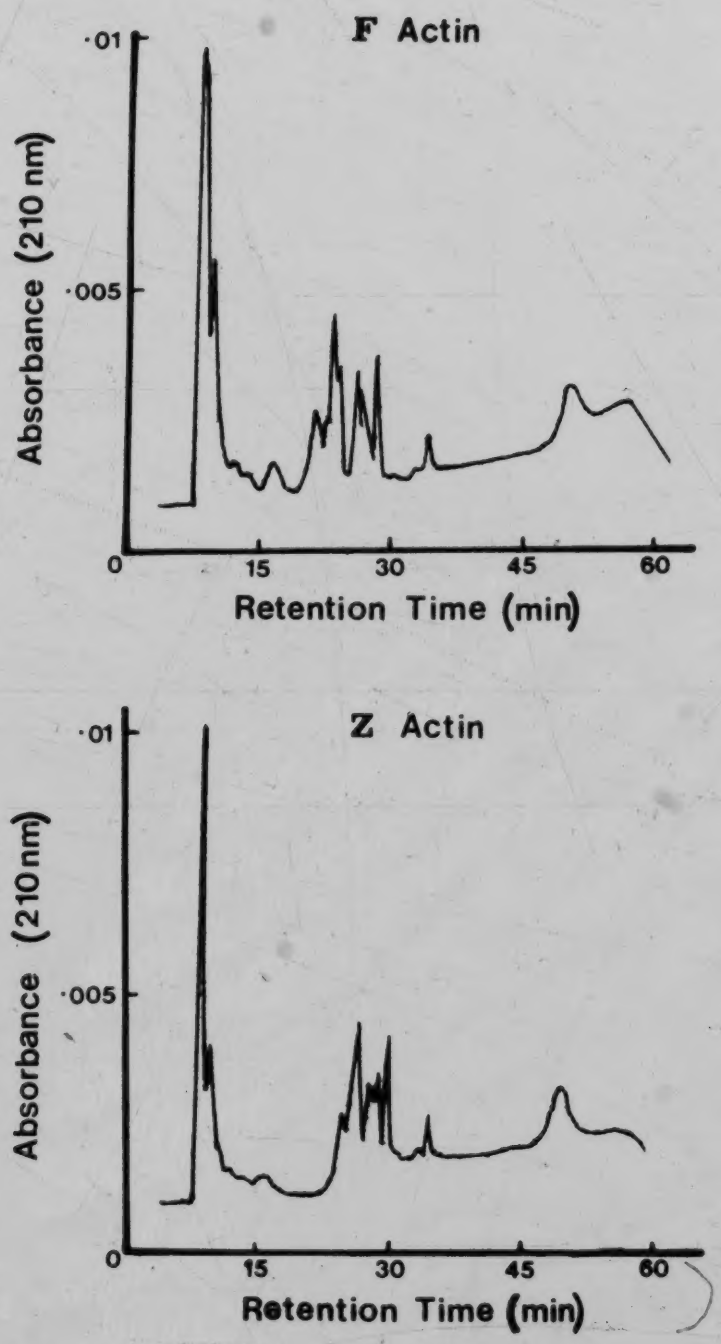

Figure 9. HPLC analysis of the 1 hour chymotryptic digest of Z actin and F actin at 210 nm. Both samples were exhaustively dialyzed in a benign buffer (appendix 2) before hydrolysis. 0.25 mg of chymotrypsin was added to 1.0 mg of the actin samples.

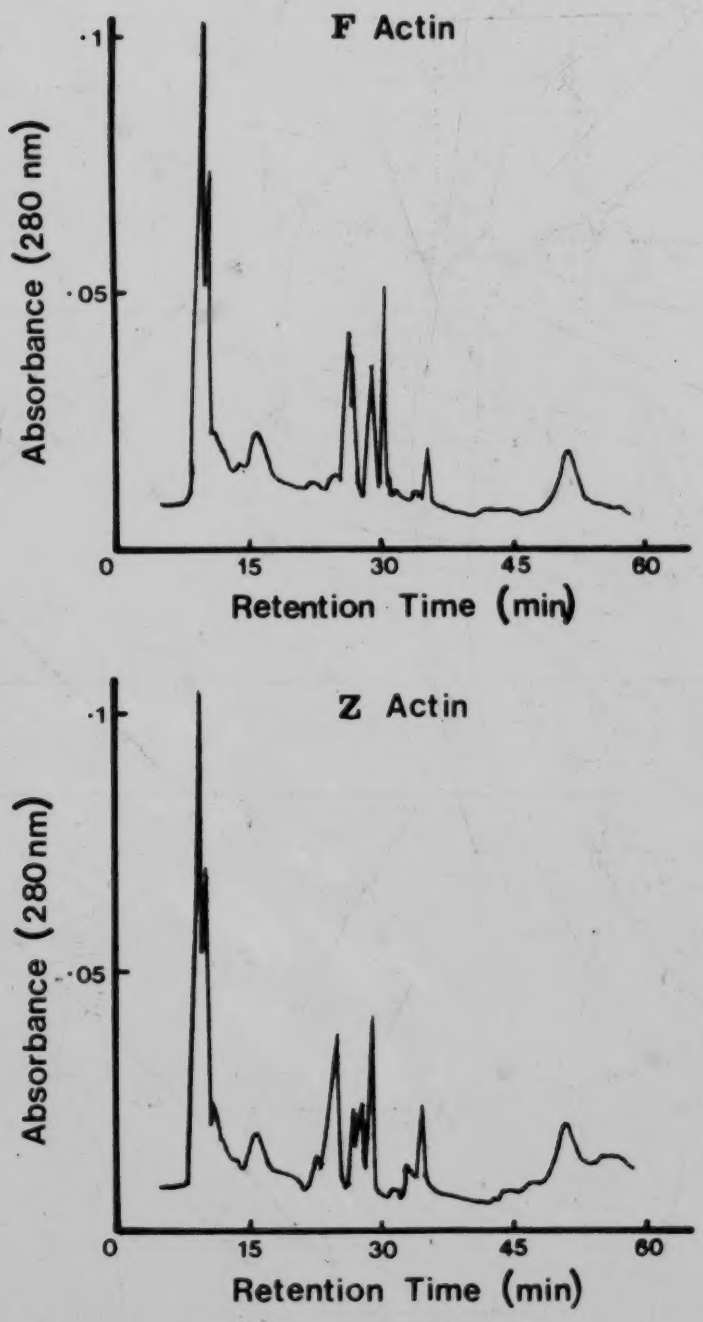

Figure 10. HPLC analysis of the 1 hour chymotryptic digest of Z actin and F actin at 280 nm. Both samples were exhaustively dialyzed in a benign buffer (appendix 2) before hydrolysis. 0.25 mg of chymotrypsin was added to 1.0 mg of the actin samples.

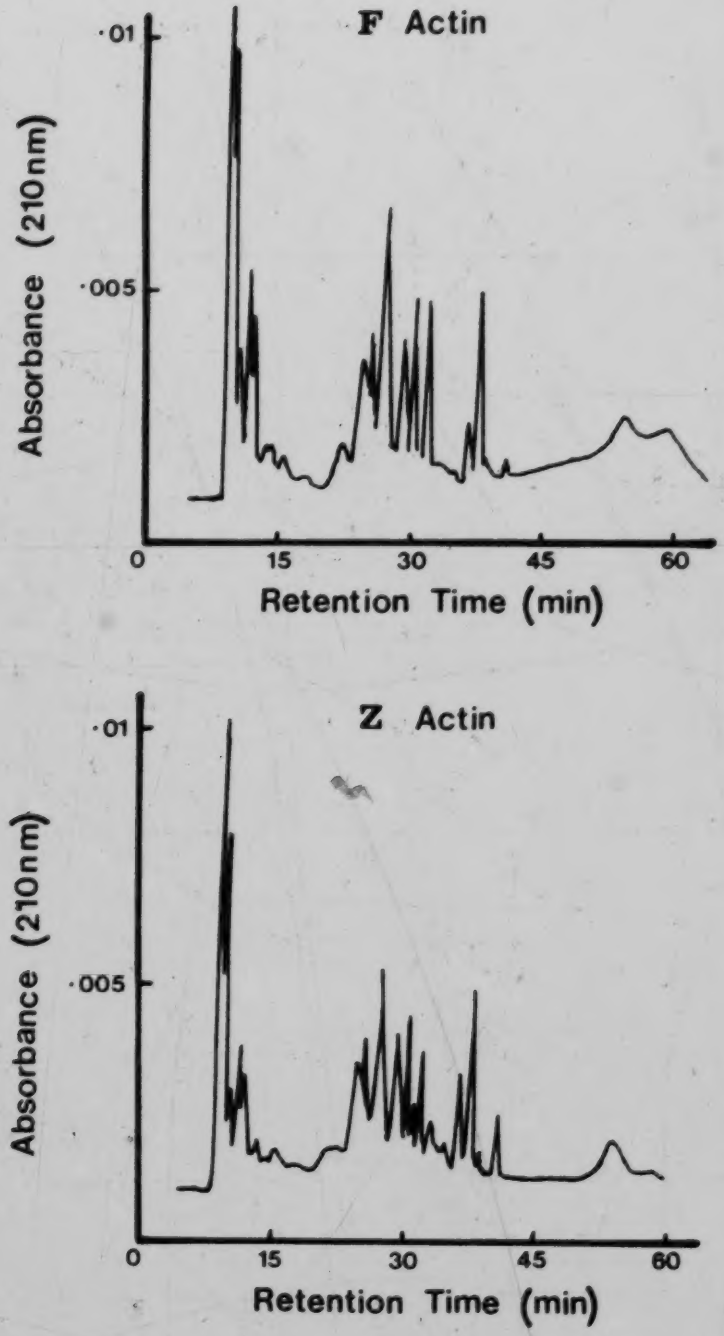

Figure 11. HPLC analysis of the 22 hour chymotryptic digest of Z actin and F actin at 210 nm. Both samples were exhaustively dialyzed in a benign buffer (appendix 2) before hydrolysis. 0.25 mg of chymotrypsin was added to 1.0 mg of the actin samples.

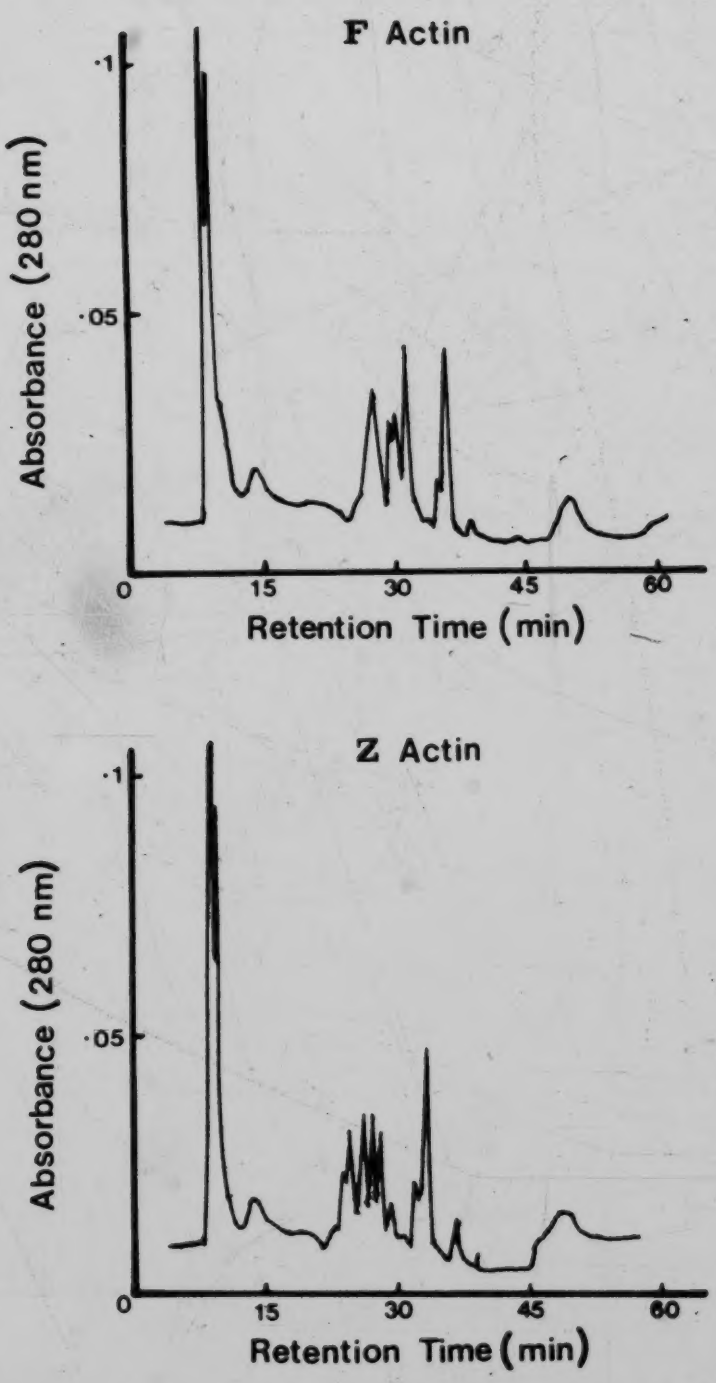

Figure 12. HPLC analysis of the 22 hour chymotryptic digest of Z actin and F actin at 280 nm. Both samples were exhaustively dialyzed in a benign buffer (appendix 2) before hydrolysis. 0.25 mg of chymotrypsin was added to 1.0 mg of the actin samples.

2.0 ml of the actin solutions were placed in stirring cuvettes and the absorbance of each solution at 340 nm was monitored. The protein concentration of each solution was kept constant at 0.4 mg/ml. The addition of 3, 30 µl aliquots of 0.33M $CaCl_2$ at 2 min intervals brought the overall $CaCl_2$ concentration to 15mM. The addition of the $CaCl_2$ brought about a large, rapid increase in absorbance in all the actin samples. The ATP concentration was increased by the addition of 5, 30 µl aliquots of 0.33M ATP until the overall ATP concentration was 25mM. This was added at 2 min intervals to initiate a clearing of the actin solutions.

Freshly purified samples of F actin and Z actin were placed in the cuvette and an increase in absorbance was observed after the addition of $CaCl_2$. As illustrated in Figure 13, addition of ATP to the untreated F actin sample initiates a much more rapid decrease in absorbance than in the untreated Z actin sample.

Half of the samples of F actin and Z actin were subjected to dialysis in 6M urea. These samples, plus the undenatured Z actin and F actin samples were exhaustively dialyzed in a benign buffer (appendix 2). As before, the absorbance of all samples was monitored at 340 nm with stirring during the addition of $CaCl_2$ and ATP. The results of these trials are illustrated in Figures 14 and 15. The F actin and Z actin samples denatured in 6M urea, as well as the exhaustively dialyzed F and Z actin solutions, behaved similarly to the freshly purified Z actin sample in the presence of $CaCl_2$ and ATP. The decrease in absorbance due to the addition of ATP to the denatured and extensively dialyzed Z and F actin samples and the freshly purified Z actin samples

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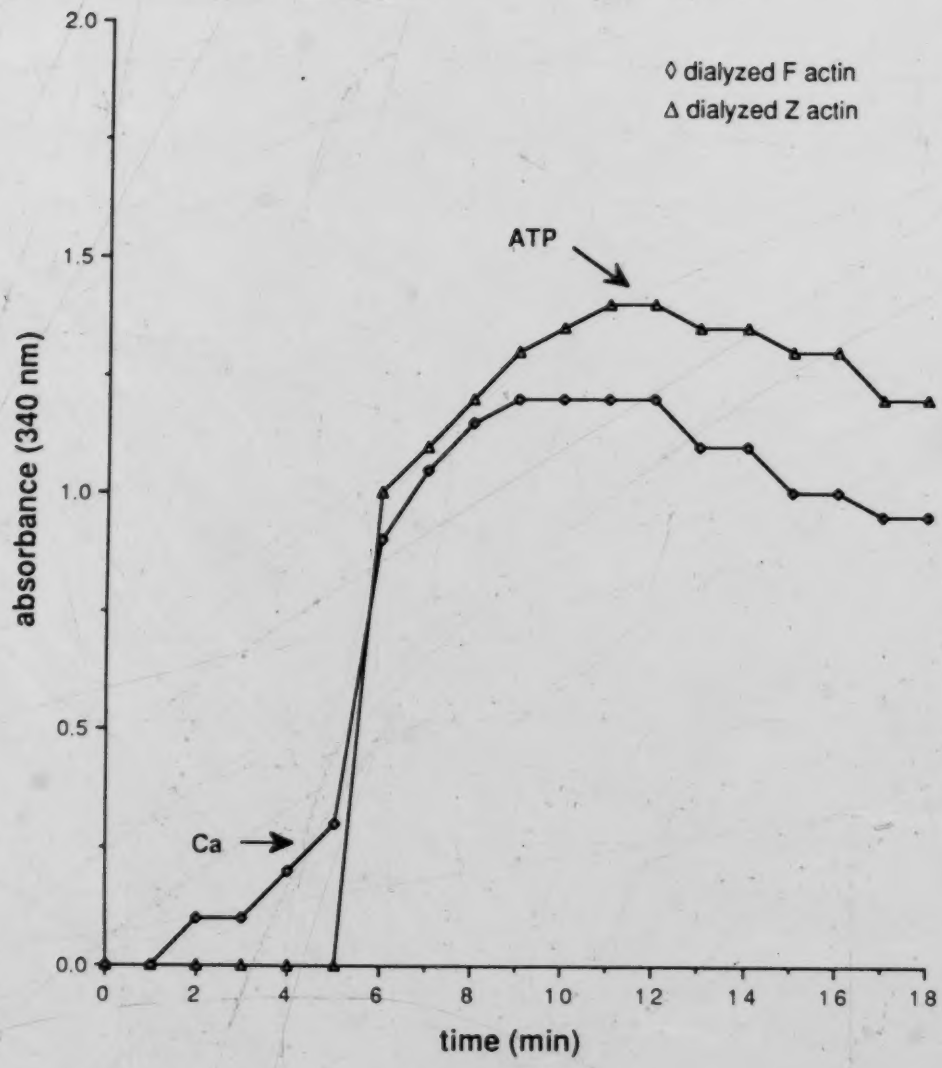

Figure 15. Aggregation of dialyzed Z actin and F actin in the presence of $CaCl_2$ and ATP. Both samples were exhaustively dialyzed in a benign buffer (appendix 2) prior to analysis. Addition of 15mM $CaCl_2$ led to a similar increase in absorbance in both samples and addition of 20mM ATP also initiated analogous responses in both solutions. Protein concentration was 0.4 mg/ml and solutions were stirred at all times.

was not as rapid and complete as in the untreated F actin. This similarity suggests that Z actin, denatured and exhaustively dialyzed F actin are virtually identical.

SUPERPRECIPITATION OF Z DISC ACTIN AND THIN FILAMENT ACTIN IN THE PRESENCE OF MYOSIN

Superprecipitation is the phenomena whereby F actin and myosin aggregate *in vitro*, creating an increase in viscosity and absorption of the solution. An attempt was made to compare the superprecipitation behaviour of denatured and exhaustively dialyzed Z and F actin in the presence of myosin. In the first 2 trials, 1.5 ml of freshly purified F actin and Z actin were added to 0.5 ml myosin and the absorbance was monitored at 340 nm while the solutions were stirred. The protein concentration of the F actin, Z actin and myosin solutions was 0.4 mg/ml. The change in absorbance of the actin-myosin suspensions was recorded while the concentrations of ATP were increased from 0 to 20mM in 5mM increments by the addition of 30 µl of 0.33M ATP at 2 min intervals. Figure 16 illustrates the change in absorbance due to the interaction of myosin and untreated F and Z actin in the presence of ATP. The last 4 trials were carried out using denatured and exhaustively dialyzed Z and F actin samples. Figures 17 and 18 illustrate their behaviour in the presence of myosin and ATP. The untreated F actin sample exhibits a different absorption pattern than the other samples. Together, the untreated F actin and myosin cause a large increase in absorbance due to the superprecipitation phenomena. Upon addition of ATP, the absorbance of the solution drops dramatically due

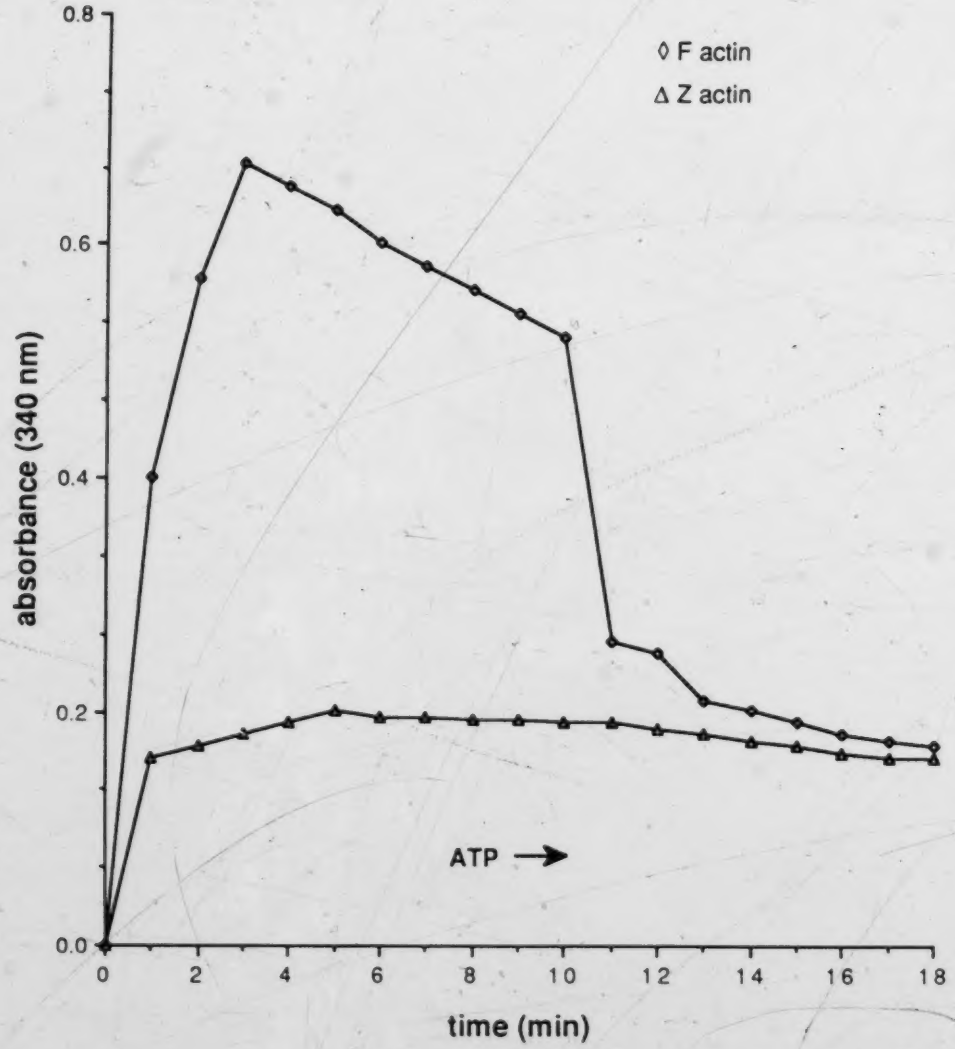

Figure 16. Superprecipitation of freshly purified F actin and Z actin in the presence of myosin. Notice how the addition of myosin caused a large increase in absorbance in the F actin sample and a much smaller increase in absorbance in the Z actin sample. 20mM ATP brought about a much more dramatic decrease in absorbance in the F actin sample compared to the Z actin-myosin solution. 0.4 mg of myosin was added to 1.2 mg of Z and F actin with constant stirring.

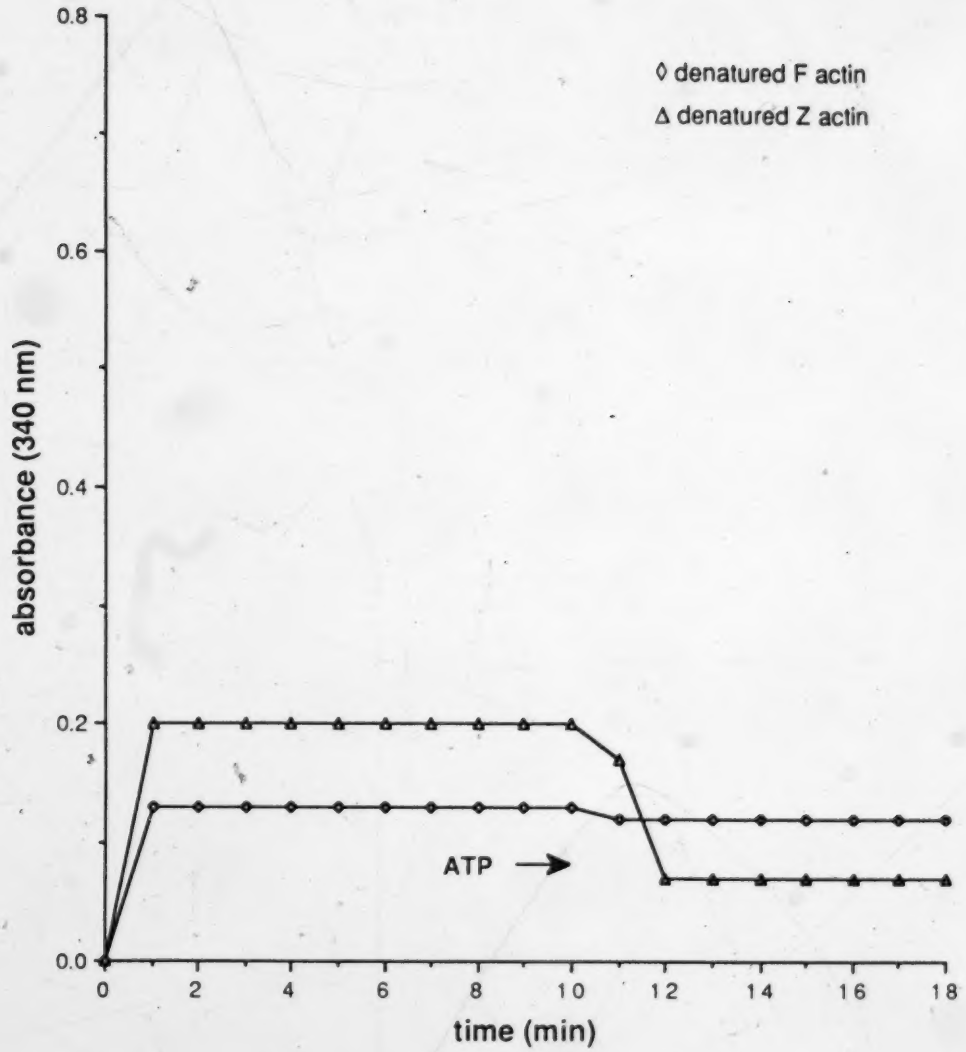

Figure 17. Superprecipitation of denatured F actin and Z actin in the presence of myosin. There was a slight increase in absorbance in the actin solutions after the addition of myosin. Absorbance stabilized in both cases after a period of 1 minute. Addition of 20mM ATP brought about a slight decrease in absorbance in the denatured F actin solution and a more pronounced decrease in absorbance in the denatured Z actin sample. 0.4 mg of myosin was added to 1.2 mg of Z and F actin with constant stirring.

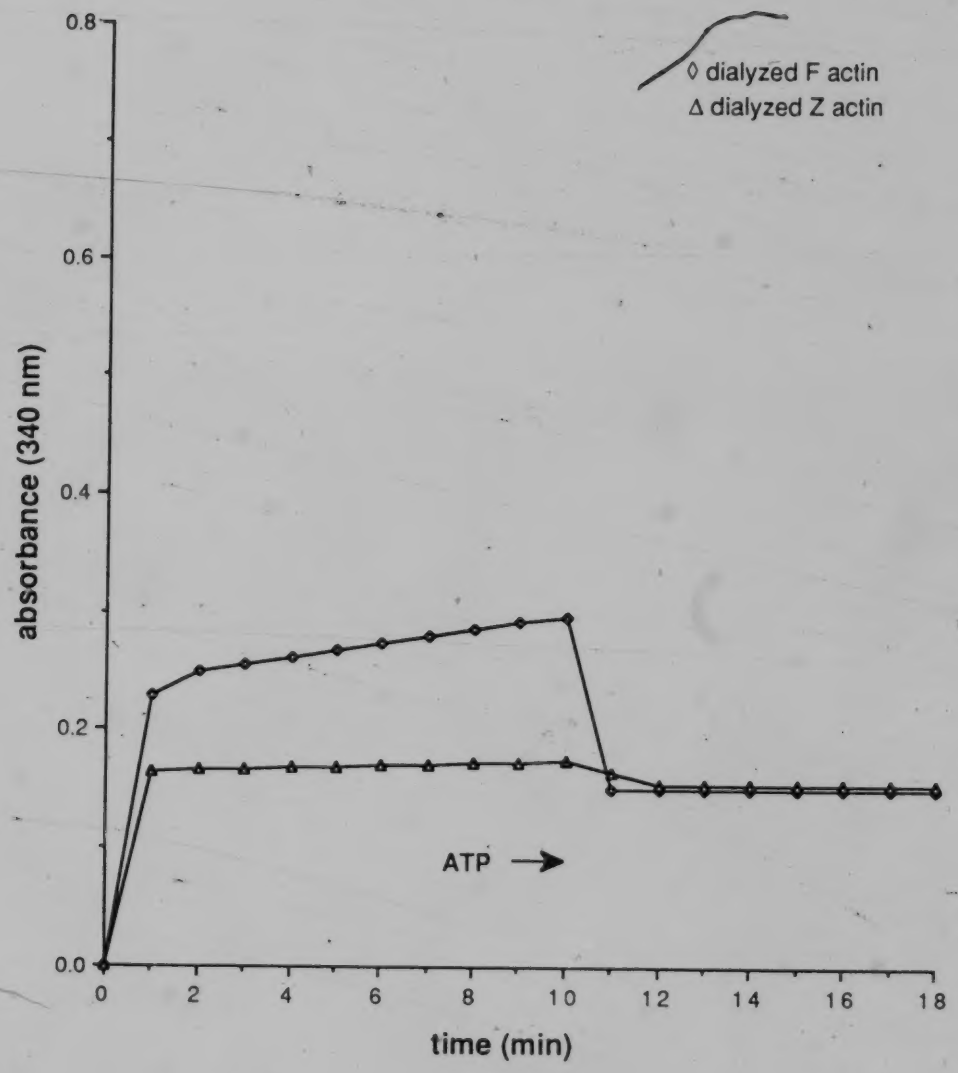

Figure 18. Superprecipitation of F actin and Z actin after dialysis in a benign buffer (appendix 2) in the presence of myosin. The addition of myosin brought about an initial increase in absorbance in both samples and a slight increase in absorbance of dialyzed F actin thereafter. Addition of 20mM ATP led to a clearing in the F actin-myosin solution and had virtually no effect on the Z actin sample. 0.4 mg of myosin was added to 1.2 mg of Z and F actin with constant stirring.

to the dissociation of actomyosin complex. Presumably the ATP allows the F actin and myosin to slide past each other much like in living, relaxed myofibrils. However, once the F actin is denatured or simply dialyzed for a period of time, it loses this ability and exhibits the same absorption pattern as the treated and untreated Z actin samples.

ELECTRON MICROSCOPY

The first successful attempt to remove the Z lines from myofibrils was accomplished by Stromer et al. (1967a). They discovered that extraction of glycerinated muscle bundles in 2mM Tris-HCl, pH 7.6 and 1mM DTT (LISB) would remove the Z lines. These researchers found that by adding the LISB extract back to the myofibres, the Z lines could be reinstated. Identification of the protein(s) responsible for this phenomena was not attempted at that time by these researchers.

Removal of the Z Lines

Complete removal of the Z discs from myofibrils proved to be much more difficult than originally hoped. The first attempt to extract the Z lines involved using LISB, as described by Stromer et al. (1967a). Although their procedure was duplicated, analysis of the electron micrographs revealed that all the Z lines remained intact.

Busch et al. (1972) used the endogenous proteolytic enzyme, CANP, to remove the Z lines from myofibrils. Using a modification of this method, a second attempt was made to remove the Z lines from myofibrils. Eight out of nine muscle bundle samples were placed in a buffer containing 10mM Tris-HCl, pH 7.4 and 10mM $CaCl_2$. The last

sample was left in the glycerol storage buffer (appendix 2) to be used as a control. Plate 6 is an electron micrograph of a myofibril with its ultrastructure intact. The muscle bundles were incubated with 18 µg of CANP for 2 hr at 25°C with gentle stirring. After digestion, the enzyme solution was removed and the samples were agitated for 1 hr in LISB (appendix 2). One muscle bundle sample was placed in the glycerol storage solution (appendix 2) to be used as an enzyme treated control. Plate 7 is an electron micrograph of a portion of a myofibril that had its Z discs removed due to the action of CANP. This experiment proved to be more successful than the Stromer et al. (1967a) method. However, detailed analysis of the electron micrographs revealed that only 5 to 10% of the Z lines were successfully extracted and as well, thick filaments were hydrolyzed in several sarcomeres. The breakdown of the thick filament caused unravelling of the myofibrils which made interpretation of the electron micrographs difficult. Plate 8 is an electron micrograph of a myofibril illustrating the unravelling of the thick and thin filaments during CANP hydrolysis and subsequent agitation.

Reconstitution of the Z Lines

An attempt was made to reconstitute the Z lines of the CANP treated myofibrils using a combination of various proteins. Z actin, F actin, alpha-actinin and LISB extract were incubated with the myofibril samples at 4°C in 0.1M KCl and 10mM Tris-HCl, pH 7.4. A total of 1.0 mg of each protein was used in all trials. Plate 9 is a photograph of an SDS-polyacrylamide gel of the various proteins used in the

Plate 6. Electron micrograph of a myofibril with its ultrastructure intact. The muscle fibril has easily discernible Z lines, H zones, A bands and I bands. (magnification = 26,000x)

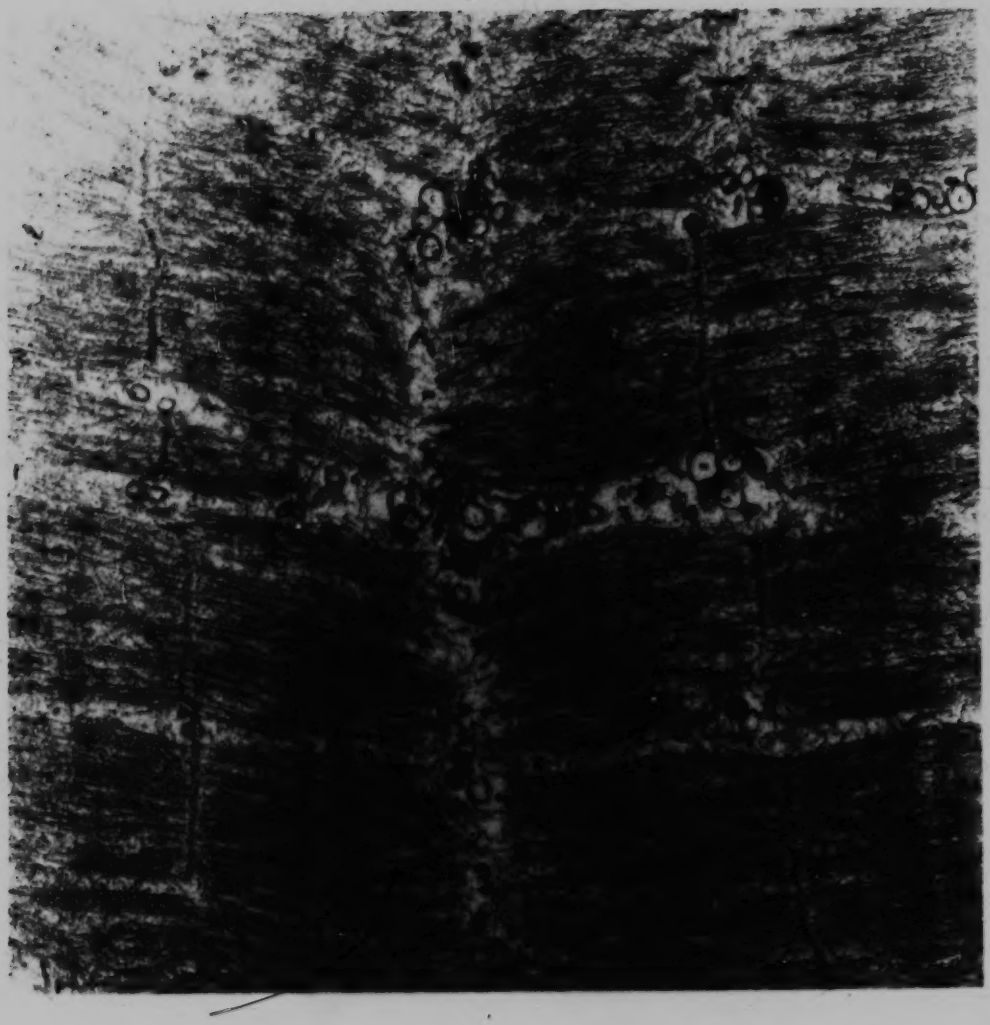

Plate 7. Electon micrograph of a myofibril treated with CANP and 10mM $CaCl_2$. Addition of the enzyme resulted in only 10 to 15% of the Z lines to be removed. (magnification = 36,000x)

Plate 8. Electron micrograph of a myofibril treated with CANP and 10mM $CaCl_2$. Aggitation of the muscle bundles during hydrolysis resulted in the unravelling of the thick and thin filaments. (magnification = 19,000x)

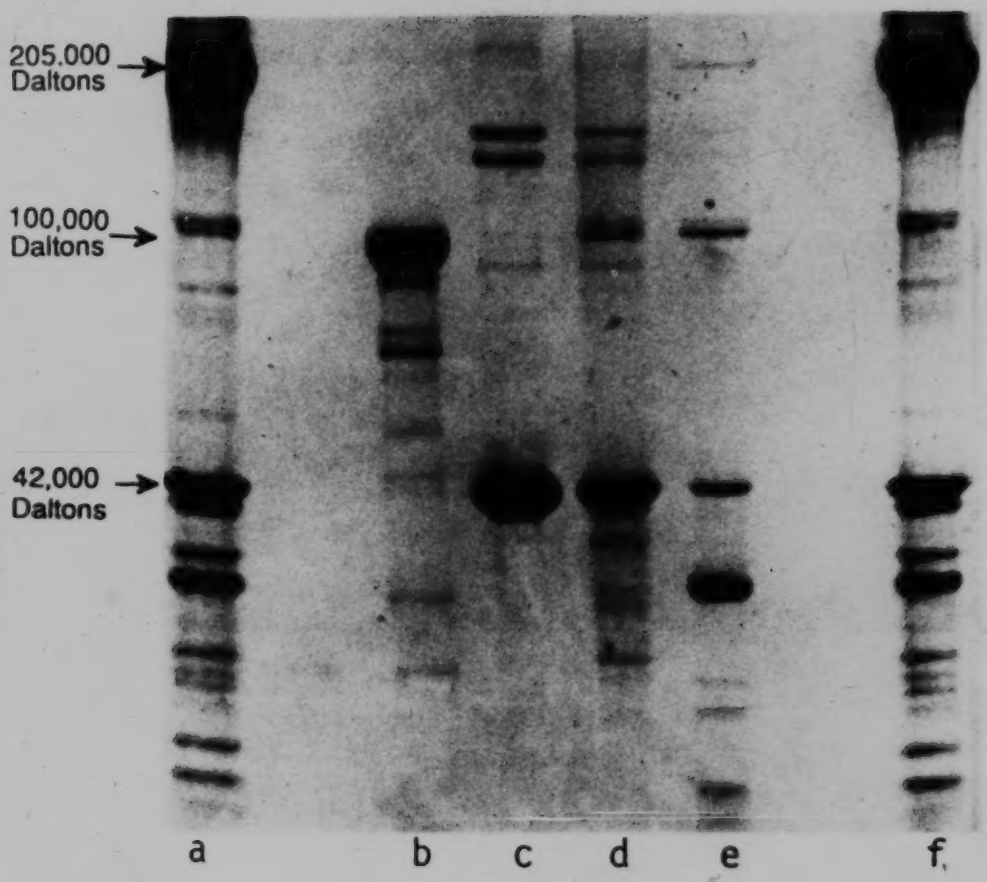

Plate 9. SDS-PAGE illustrating the various proteins isolated for the attempted reconstitution of the Z line in myofibrils. Lanes (a) and (f) are standard myofibril solutions; (b) alpha-actinin; (c) F actin; (d) Z actin and; (e) LISB extract of a myofibril solution.

attempted reconstitution of the Z lines of myofibrils. Table 2 illustrates the combination of proteins used:

Table 2 : Proteins used in the attempted reconstitution of the Z line of myofibrils.

1. Treated with CANP.
2. Alpha-actinin.
3. Alpha-actinin and Z actin.
4. Z actin.
5. Alpha-actinin and F actin.
6. F actin.
7. Alpha-actinin, F actin and Z actin.
8. F actin and Z actin.
9. No extraction.

Although the isolated proteins were incubated with the muscle bundles for 66 hr as planned, interpretation of the electron micrographs proved difficult due to incomplete removal of the Z lines.

SDS-PAGE ANALYSIS OF THE CANP DIGESTION OF Z DISC ACTIN AND THIN FILAMENT ACTIN

The susceptibility of Z actin to hydrolysis by CANP has been plainly demonstrated by Nagainis and Wolfe (1982). That work also revealed that thin filament actin is resistant to degradation by this enzyme. Clearly there is a difference between the proteins which allows F actin to resist hydrolysis by CANP while Z actin is rapidly broken down by this enzyme.

Half of the Z actin and F actin samples were denatured in 6M urea and then the denatured and undenatured proteins were dialyzed in a benign buffer (appendix 2). The samples were incubated with CANP in a 5mM $CaCl_2$ buffer for 30 min at 25°C. After hydrolysis, the protein samples were analyzed using SDS-PAGE to yield the results shown in

Plate 10. This gel clearly demonstrates that dialyzed denatured and undenatured F actin exhibits the same susceptibility to enzymatic digestion by CANP as the treated and untreated Z actin samples. However, F actin that has not been subjected to dialysis remains resistant to hydrolysis by this enzyme. Although not shown here, extending the extraction time of F actin from 1 hr to 24 hr produced a protein that was quickly hydrolyzed by CANP. Altering the extraction times of Z actin from 1 hr to 24 hr did not alter the susceptibility of Z disc actin to hydrolysis by CANP. After depolymerization and repolymerization of Z actin and exhaustively dialyzed F actin, there was a slight loss in the ability of CANP to hydrolyze Z actin. This treatment had no effect on the exhaustively dialyzed thin filament actin, as it still exhibited its susceptibility to hydrolysis by CANP. The possibility exists, therefore, that denaturing or exhaustively dialyzing thin filament actin somehow affects the conformation of the molecule which changes the susceptibility of F actin to hydrolysis by CANP.

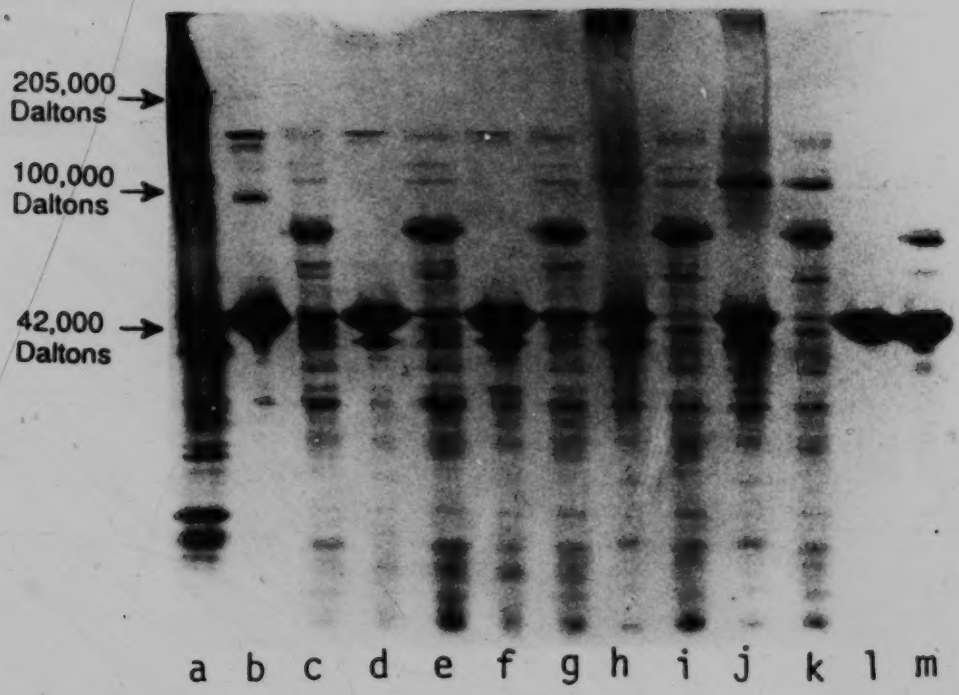

Plate 10. SDS-PAGE demonstrating the effect of the addition of CANP and 6mM $CaCl_2$ to various samples of F actin and Z actin. Denatured samples were dialyzed in 6M urea and then dialyzed in a benign buffer (appendix 2). Dialyzed samples were simply dialyzed in the benign buffer. (a) standard myofibril solution; (b) freshly purified Z actin; (c) Z actin and CANP; (d) denatured Factin; (e) denatured F actin and CANP; (f) dialyzed F actin; (g) dialyzed F actin and CANP; (h) denatured Z actin; (i) denatured Z actin and CANP; (j) dialyzed Z actin; (k) dialyzed Z actin and CANP; (l) freshly purified F actin; (m) F actin and CANP. 5 ug of CANP was added to 35 ug of sample and allowed to react for ½ hour at 25 degrees Celcius.

DISCUSSION

Research was undertaken to determine and elucidate the differences between Z disc actin and thin filament actin. Although freshly purified F actin and Z actin behaved differently in several experiments *in vitro*, these proteins exhibit homologous behaviour after thin filament actin was dialyzed for an extended period of time in a non-denaturing buffer. During the isolation procedure for Z actin, one of the purification steps involves extracting myofibrils for 24 hr with LISB. This lengthy extraction may be responsible for the loss of the loosely bound nucleotide from thin filament actin. One of three possibilities may explain the existence of Z disc actin.

1. Thin filament actin may be extracted from the myofibrils. During its removal, ADP may be lost, yielding an actin moiety that is partially denatured.

2. Z disc actin may have a slightly different primary structure than thin filament actin. Since it is known that there are several isoforms of F actin, Z actin may be a unique isoform of thin filament actin residing in the Z disc of myofibrils. This may explain some of the behavioural differences between the two proteins.

3. Z disc actin may be structurally identical to thin filament actin. During the construction of the myofibril, F actin may be incorporated into the Z line, void of the ADP molecule. This may be a necessary prerequisite which slightly alters the conformation of the protein, enabling it to interact with other proteins in maintaining the ultrastructure of the Z line.

Experimental results presented here indicate that Z disc actin is probably a denatured form of thin filament actin. The denaturation of F actin may occur concomitant to the loss of the ADP molecule during the extended extraction period.

Research by Straub and Feuer (1950) revealed that ADP is noncovalently bound to thin filament actin *in vivo*. Exhaustive dialysis in 0.1M KCl and denaturation with urea have both been used to remove the nucleotide from F actin (Mommaerts, 1952; Kasai et al., 1965). Mommaerts found that the removal of ADP from thin filament actin seems to make the molecule unstable, probably due to a change in conformation of the protein.

During the polymerization of globular (G) actin to thin filament (F) actin in the presence of ATP, the ATP is hydrolyzed to ADP and inorganic phosphate. The ADP binds stoichiometrically to the individual actin molecules in the filament, while the inorganic phosphate is released. The polymerization of G actin to F actin filaments can take place spontaneously in the presence of monovalent cations in concentrations from 50 to 100mM. However, ADP bound to the G monomers is essential to this process *in vivo*. The monovalent salts are believed to change the conformation of the actin monomers, allowing them to aggregate into the double stranded alpha-helical actin filament. Martonosi et al. (1964) found that divalent cations such as Ca^{++}, Mg^{++} and Mn^{++} must be bound in a 1:1 molar ratio before the process of polymerization can take place. It was later revealed by Golaszlioska et al. (1978) that actin has at least one high affinity divalent cation binding site. These researchers also suggested that actin may have as

many as 5 other cation binding sites existing on the molecule. They concluded that at least 4 of these sites must be occupied before the electrostatic repulsion is low enough to allow aggregation of the actin molecules prior to polymerization. Although this transformation can proceed without the addition of divalent cations in vitro, their presence initiates a more rapid polymerization of the G actin monomers than with monovalent cations only. The presence of ATP is thought to stabilize the F actin filaments and prevent aggregation of the filaments leading to precipitation during storage.

Denaturation in 6M urea was used to unfold Z actin and F actin. These proteins were then dialyzed in a benign buffer and various experiments were carried out to determine if the behaviour of the proteins would be homologous. Although some proteins do not totally unfold in high concentrations of urea, the majority of proteins will undergo substantial denaturation in this solvent. Urea interrupts the hydrogen bonding on the surface of proteins and alters hydrophobicity by exposing the interior of the molecule. X-ray crystallography studies on several enzymes have shown that denaturation by urea can be reversible, provided the proper solvent conditions are maintained during the renaturation process (Lapanje, 1978). Renaturation is said to occur when the restoration of the 3 dimensional structure takes place, resulting in a resumption of biological activity.

ADP has an absorption maxima at 259 nm, due to the presence of the adenine ring. Proteins, on the other hand, absorb strongly between 190 and 220 nm and at 280 nm due to the presence of the peptide bonds and aromatic amino acids, respectively. As revealed previously (Figure

5) the results of the scanning UV spectrophotometry analysis of F actin showed a 260 nm peak which vanished after exhaustive dialysis or denaturation (Figures 6 and 7). Since it is known that 1 mole of thin filament actin noncovalently binds 1 mole of ADP, it is not surprising to discover a loss of the 260 nm peak after these treatments. Furthermore, analysis revealed that freshly purified Z disc actin does not contain the 260 nm peak. This suggests that some of the behavioural differences between Z actin and F actin may be due to the presence of the bound ADP. As mentioned before, (Korn, 1982), the loss of this charged molecule could affect the conformation of F actin, possibly yielding a protein that exhibits characteristics which are slightly altered.

Chymotrypsin is a serine endopeptidase that has the ability to cleave peptides, amides and esters. This enzyme preferentially hydrolyzes carboxyl groups of hydrophobic amino acids such as tryptophan, tyrosine, phenylalanine and leucine. Chymotrypsin was incubated with exhaustively dialyzed F actin and Z actin for 1 hr and 22 hr. Since chymotrypsin hydrolyzes proteins at fairly specific cleavage sites, it would be expected that the hydrolysis of proteins with similar conformations would yield similar peptides. The action of this enzyme on dialyzed F actin and Z actin produced reverse-phase HPLC chromatograms that are very similar, if not identical (Figures 9 to 12). It seems reasonable to conclude that the peptides released during proteolysis were analogous in MW and hydrophobicity since the peak heights and retention times are similar. Although the tertiary structure of Z disc actin has not been determined, these results strongly suggest

that Z actin and extensively dialyzed F actin exhibit conformational homology.

The loss of a ligand may affect the conformation and functionality of a protein. The removal of ADP from thin filament actin destabilizes the protein, reversibly altering its conformation (Korn, 1982). Attempts to convert exhaustively dialyzed or denatured thin filament actin and Z actin to G or F actin by addition of ATP proved unsuccessful. Figure 19 illustrates the possible relationship between Z actin and F actin and demonstrates the method of isolation of each protein. Although Z actin was polymerized in the presence of ATP, analysis in a scanning UV spectrophotometer revealed that polymerized Z actin did not absorb at 260 nm. Depolymerization and repolymerization of dialyzed F actin and freshly purified Z actin in the presence of ATP yielded proteins still susceptible to hydrolysis by CANP, although Z actin seemed to be somewhat more resistant to the proteinase than before. The tertiary structure of a protein can be affected by numerous other physical and chemical factors. Inorganic salts are known to cause conformational changes in proteins, possibly leading to denaturation (for a review see Lapanje, 1978). Salts can exhibit a nonspecific effect on proteins by screening out interactions between charged moieties on the peptide chain. The presence of divalent cations may change the conformation of the protein, affecting the way it interacts with the solvent and other proteins. This change may be detected by methods such spectrophotometry and viscosity. In certain cases, denatured proteins may become insoluble in the medium and precipitate out of solution. This precipitation causes an increase in absorption

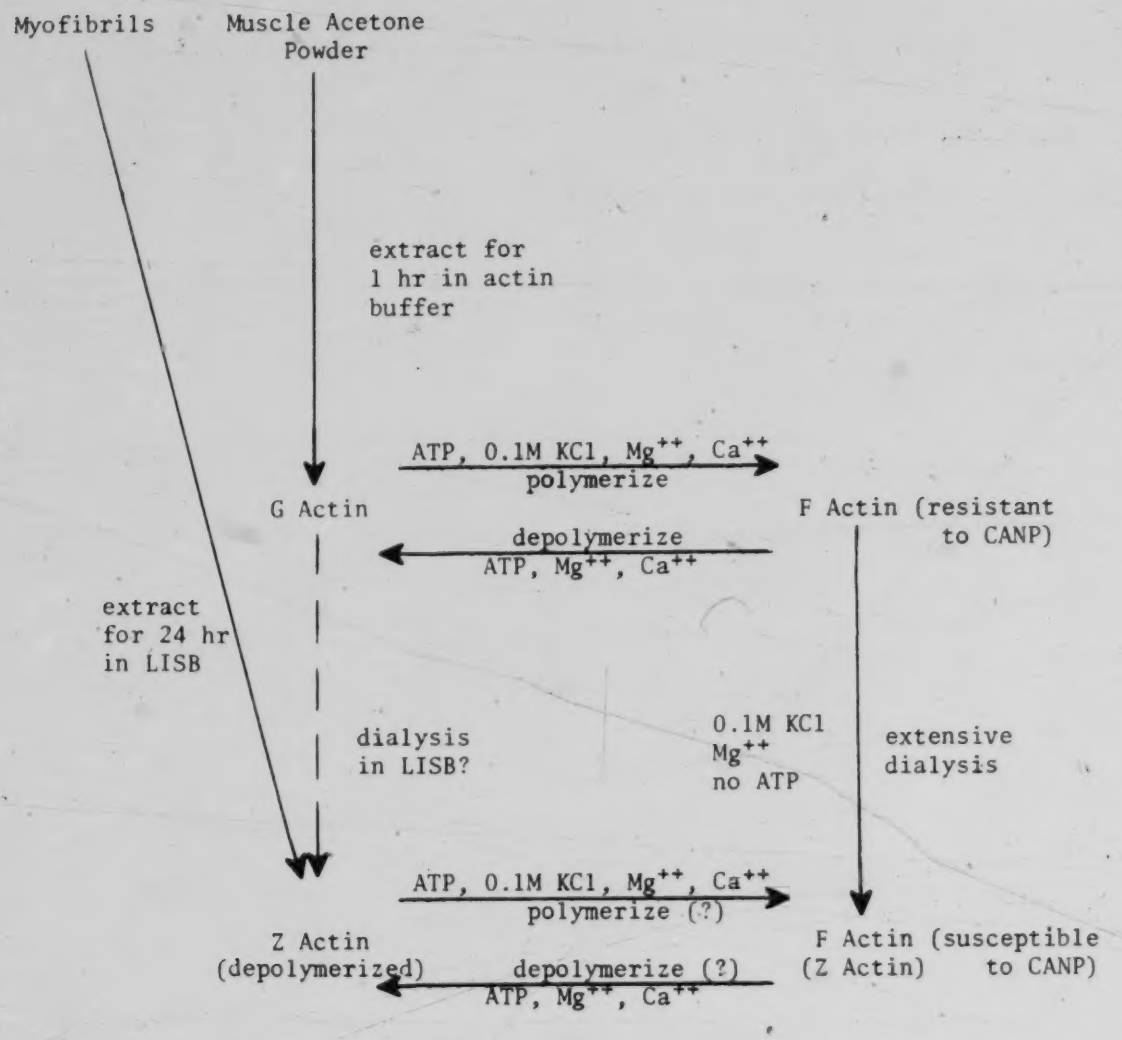

Figure 19. Flow chart illustrating the possible relationship between Z actin and F actin.

due to the presence of insoluble particles in solution. The extent to which salts interact with proteins depends on the number of charged species on the outside of the molecule accessible to the ions. A change in conformation of a protein can alter the number and strength of electrostatic charges available for the interaction with the salt.

In high concentration, $CaCl_2$ may cause denaturation resulting in precipitation of proteins such as F actin. However, bringing the $CaCl_2$ concentration to 15mM in the Z actin and F actin samples caused a large increase in absorbance at 340 nm (Figures 13 to 15). This increase in absorbance was probably due to the aggregation of actin molecules. There is little chance that G --> F transformation-type polymerization caused the increase in absorbance. This is due to the fact that at the ionic strength of the buffer used (0.1M KCl), actin was probably in the filamentous form and therefore already polymerized. Researchers have found that actin is in the filamentous form at this ionic strength as detected by a change in UV absorption at 232 nm (Mockrin and Korn, 1981). The addition of ATP brought about a slow decrease in absorption in the dialyzed denatured and undenatured Z actin and F actin samples. However, a large rapid decrease in absorbance was observed in the freshly purified F actin sample upon inclusion of ATP (figures 13 to 15). This event may be explained by the way the negatively charged ATP molecules electrostatically interact with the proteins and cations in solution. ATP seems to either remove or mask the insolubility caused by the protein-cation complex much more quickly with freshly purified F actin than the other samples. The presence of ATP may bring about a change in conformation of F actin which allows

it to become soluble much more quickly than the dialyzed denatured and undenatured Z and F actin samples.

Under certain conditions, thin filament actin and myosin will form a complex in solution causing an increase in turbidity and viscosity. This phenomena, called superprecipitation, is affected by ionic strength and the presence or absence of ATP. In conditions of high ionic strength (~0.6M KCl), and no ATP, actin and myosin will aggregate together causing a precipitate. Remove the ATP, however, and the solution clears due to the dissociation of the actin and myosin. At physiological ionic strength (~0.1M KCl), the actomyosin solution becomes turbid in the absence of ATP. Addition of ATP at this time brings about a clearing of the solution first described by Szent-Gyorgyi (1949). As the ATP concentration decreases, a gel will form indicating that superprecipitation has occurred. It is generally accepted that the clearing of the actomyosin solution coincides with muscle relaxation and that superprecipitation coincides with muscle contractions in vivo.

Several mechanisms have been proposed to explain the interaction of thin filament actin and myosin during superprecipitation and muscle contraction (for a review see Schmalbruch, 1985). The conformation of the actin molecule must be acceptable to the myosin S-1 subunit if binding is to occur. This conformation must somehow be altered during the dialysis and denaturation of thin filament actin. Figure 16 illustrates the superprecipitation of F actin and myosin in a 0.1M KCl solution in the absence of ATP. As expected, the addition of ATP resulted in the dissociation of the actomyosin complex and a clearing

of the solution. Figures 17 and 18 show the change in absorbance of the dialyzed denatured and undenatured Z and F actin samples and the freshly purified Z actin in the presence of myosin and ATP. Although a slight increase in absorbance was observed with these samples upon addition of myosin, it was only a fraction of the increase observed with untreated F actin. Since the treated F actin samples behaved similarly to freshly purified Z actin in the presence of myosin and ATP, the removal of the 260 nm absorbing compound may change its tertiary structure.

The removal and reconstitution of the Z lines of myofibrils proved to be less successful than hoped. Although this experiment was successfully carried out by Stromer et al. (1967a), a recent discussion with one of the authors, Hartshorne (1987, pers. comm.), revealed that several subsequent attempts to reproduce Stromer's work were not fruitful. One can only speculate as to the reasons why this experiment can not be successfully duplicated.

Although the removal of the Z lines using CANP proved to be more successful, severe unravelling of some of the thick and thin filaments made interpretation of the electron micrographs difficult (Plate 8). Agitation of the muscle bundles might have caused the myofilaments to become randomly scattered in the myofibril. This fact, coupled with the incomplete removal of the Z lines, made the attempted reconstitution of the Z discs almost impossible to evaluate.

Reconstitution of the Z line was successfully accomplished by Stromer et al. (1967a). These researchers extracted the Z lines of rabbit psoas muscle using LISB. The extract was then subjected to

ammonium sulphate fractionation and the 40% precipitate was solubilized in LISB. These researchers found that the Z lines could be reconstituted with the LISB extract and the solubilized proteins in the 40% ammonium sulphate precipitate. However, they failed to identify the proteins responsible for the reconstitution.

CANP treated myofibrils in our experiment were incubated with the various combinations of alpha-actinin, F actin and Z actin. Conclusions could not be drawn, however, due to the fact that more widespread removal of the Z lines occurred during the extraction process. Several researchers have successfully removed the Z lines of myofibrils using CANP (for a review see Goll et al., 1983a). Whether the Z disc can be reconstituted or not, using these proteins, hinges on the complete removal of the Z lines from myofibrils. Z disc actin is believed to be a major constituent of the Z line and is known to be susceptible to CANP (Nagainis and Wolfe, 1982). The natural progression is to attempt the reconstitution of the Z lines of myofibrils using Z disc actin, regardless of whether this protein is simply a denatured form of F actin or not. The information gained by the incubation of the Z disc extracted myofibrils with Z actin, F actin and alpha-actinin will be extremely fruitful. This knowledge could be used to further our understanding of the structure of the Z line.

CANP is a calcium activated neutral proteinase with a cysteine group present at its active site. As mentioned earlier, this enzyme preferentially hydrolyzes the Z line of myofibrils and Z disc actin. The action of CANP on Z actin can be quickly monitored using SDS-PAGE. Our results have confirmed that freshly purified F actin is resistant

to hydrolysis by CANP (Plate 10). It is known that either exhaustive dialysis or denaturation of F actin removes a compound absorbing at 260 nm. The removal of this loosely bound entity from F actin during these treatments may alter the conformation of the protein enough to make it susceptible to CANP . Incubation of CANP with both the dialyzed and denatured F actin samples completely hydrolyzed the proteins as revealed by SDS-PAGE. Although this evidence is not conclusive, it does imply that the difference between Z actin and F actin may simply be due to the presence of a compound absorbing at 260 nm, presumably ADP, which alters the conformation of the protein. To fully elucidate this problem, different avenues of research would have to be undertaken. Ideally, amino acid sequencing and X-ray crystallography would be used to more fully characterize the differences between Z disc actin and thin filament actin, however the equipment and expertise was not available to attempt these methods of analysis. A conclusion can still be obtained drawing from our knowledge of the composition of thin filament actin and the various analytical biochemical experiments illustrated in this report.

The presence of highly charged ligands alters the conformation of proteins which, in turn affects their biological activity. It has long been known that <u>in vivo</u>, one mole of thin filament actin contains one mole of noncovalently bound ADP. The removal of the ADP molecule from F actin probably affects its tertiary structure and alters the manner in which the protein behaves in the presence of denaturing salts and myosin. This change in conformation may also affect the susceptibility of F actin to hydrolysis by proteinases such as CANP.

It follows that the loss of the nucleotide from F actin yields a protein that has similar cleavage sites as Z actin. The HPLC elution profiles of the chymotryptic hydrolysates of the exhaustively dialyzed F and Z actin samples are analogous, indicating a possible conformational homology.

Albeit not 100% conclusive, the experimental data gathered here strongly suggests that the KI insoluble actin-like protein is merely thin filament actin devoid of its loosely bound nucleotide. Although this interpretation seems simplistic, the thought of a slightly altered form of thin filament actin present in the Z line is an interesting concept. The possibility exists that this Z disc actin could play a role in the maintenance of the integrity of the Z line and, therefore have a direct effect on the conversion of muscle to meat.

CONCLUSION

An attempt was made to determine the nature and extent of the difference, if any exist, between Z disc actin and thin filament actin. Nagainis and Wolfe (1982) showed that Z disc actin differed from thin filament actin in its solubility in 0.6M KI, susceptibility to hydrolysis by CANP and isoelectric point. These researchers also demonstrated that the two proteins have similar amino acid composition and MW, as demonstrated by SDS-PAGE.

The evaluation of Z disc actin and thin filament actin using the biochemical methods reported in this thesis revealed that these two proteins differ only by the presence of the 260 nm absorbing compound in the F actin. Results presented here suggest that Z actin is simply thin filament actin devoid of the 260 nm absorbing compound. The loss of this compound, presumably ADP, probably changes the conformation of F actin, allowing this molecule to exhibit similar behaviour to Z actin in the presence of denaturing salts and myosin. In addition, HPLC analysis of the chymotryptic hydrolysate of dialyzed F actin and Z actin revealed that the elution profiles of the proteins were similar. Freshly purified thin filament actin is resistant to proteolysis by CANP. Only after exhaustive dialysis or denaturation is F actin susceptible to hydrolysis by this proteolytic enzyme. This differed from Z actin in that freshly purified, dialyzed or denatured Z actin were all rapidly broken down by CANP.

Researchers have demonstrated that the Z line of myofibrils can be removed with LISB or CANP (Stromer et al., 1967a; Busch et al.,

1972). This evidence, together with the knowledge that Z actin is isolated from myofibrils using LISB and is susceptible to hydrolysis by CANP, suggests that Z disc actin probably performs an important function in the maintenance of the Z disc. Since it has been established that weakening of the Z line during meat ageing correlates to increased tenderness, the possibility still exists that Z disc actin plays an important role in the conversion of muscle to meat.

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APPENDIX 1

Polyacrylamide Gel Electrophoresis

The separating gels were poured with a gradient of acrylamide ranging from 7.5% to 20%. The stacking gel had an acrylamide concentration of 3%. All reagents used were electrophoresis grade purchased from Bio-Rad Industries.

Gel Preparation

The gel slabs were made from stock solutions which consisted of:

Lower Stock	: 1.5M Tris-HCl, pH 8.8 and 0.4% (w/v) SDS
Upper Stock	: 0.5M Tris-HCl, pH 6.8 and 0.4% (w/v) SDS
Acrylamide Stock	: 30% (w/v) acrylamide and 0.8% (w/v) bis-acrylamide
Ammonium Persulfate	: 10% (w/v) ammonium persulfate in distilled H_2O (prepared immediately prior to use)
Glycerol	: 60% (v/v) glycerol in distilled H_2O
N,N,N',N' tetramethylethylenediamine (TEMED)	

The separating gel consisted of the compounds mentioned above, in the following quantities :

	7.5% acrylamide	20% acrylamide
Lower Stock	4.5 ml	4.5 ml
Acrylamide Stock	4.5 ml	12.0 ml
60% glycerol	0.8 ml	1.6 ml
Distilled H_2O	8.1 ml	--
10% ammonium persulfate	30 µl	30 µl
TEMED	8 µl	8 µl
Total	18 ml	18 ml

The compounds were gently swirled and placed in a Buchler gradient mixer. The solutions were agitated with a Buchler vibrating stirrer to ensure a linear gradient. A Buchler polystaltic pump was used to draw the solution into the gel casting unit. After this was accom-

plished, 2-methyl-1-propanol was layered on top of the acrylamide to ensure that the top of the gel polymerized without a meniscus. After allowing the acrylamide to polymerize for at least one hour, the 2-methyl-1-propanol was poured off in preparation for addition of the stacking gel. The stacking gel was made up in the following manner :

Distilled H_2O	3.1 ml
Upper Stock	1.3 ml
Acrylamide Stock	0.6 ml
10% ammonium persulfate	25 µl
TEMED	5 µl
Total	5 ml

The appropriate comb was placed on the casting unit and the stacking gel was poured on the separating gel with a pasteur pipette. In 20 min the gel was fully polymerized. The protein samples were run in a buffer containing 0.2M Tris-HCl, pH 8.6, 1.92M glycine and 0.1% (w/v) SDS, diluted 10 fold before use. The gels were run at a constant current of 35 milliamps for 6 hr with the Protean II gel unit and 18 milliamps for 2 1/2 hr with the Mighty Small gel unit.

Sample Preparation for SDS-PAGE

The protein solutions to be analyzed were mixed in a 1:1 ratio with a buffer containing 0.05M Tris-HCl, pH 6.8, 1% (w/v) SDS, 30% (v/v) glycerol and 2% (v/v) ß-MCE. Bromophenol Blue (BDH) was added to this solution in a concentration of 0.01% (w/v) so the progress of the electrophoresis run could be monitored. The protein samples were mixed with the sample buffer for 2 hr at 37°C prior to application on the gel. Ten µg of protein were applied to each well to obtain the

proper sample concentration.

Molecular Weight Determinations

The molecular weight of the proteins was determined with the aid of standards. Two sets of standards were routinely used. One was obtained from the Sigma Chemical Company and contained the following proteins :

Protein	Approximate MW
Carbonic anhydrase (bovine erythrocytes)	29,000
Egg Albumin (ovalbumin)	45,000
Bovine Plasma Albumin	66,000
Phosphorylase B (rabbit muscle)	97,400 (subunit)
ß-Galactosidase (Escherichia coli)	116,000 (subunit)
Myosin (rabbit muscle)	205,000 (subunit)

The other set of standards was a myofibrillar protein solution prepared in our lab from chicken pectoral muscle. This solution contained the following major myofibrillar proteins:

Protein	Approximate MW
Tropomyosin	36,000 (subunit)
Actin	42,000
Alpha-Actinin	100,000
Myosin	205,000 (subunit)

The standards were prepared for the SDS-PAGE in the same manner used in the preparation of the unknown proteins. Molecular weights of the unknown proteins were determined from a standard curve which plotted the log of molecular weight of the standard proteins versus electrophoretic mobility.

Gel Staining and Destaining

After the electrophoretic run was completed, the gel was placed in a staining solution containing, 45% (v/v) methanol, 10% (v/v) acetic acid and 0.35% (w/v) Coomassie Blue R-250 (Serva) and gently agitated on a Junior Orbit Shaker (Lab-Line Instruments Inc.). After the protein bands were allowed to stain for at least 8 hr, the gel was placed in a destaining solution containing 30% (v/v) methanol and 10% (v/v) acetic acid. Several changes of destaining solution were required to remove background stain. The gel was placed between cellophane sheets on a drying assembly and allowed to dehydrate overnight as outlined in the procedure of Wallevik and Jensenius (1982). The gels were stored as thin sheets and photographed as required.

APPENDIX 2

Buffers and Their Chemical Composition

myofibril buffer	:	0.1M potassium chloride (KCl), 20mM potassium phosphate, pH 6.8, 2mM magnesium chloride ($MgCl_2$) and 2mM ethylene-glycol-bis (ß-aminoethyl ether) N,N'-tetra acetic acid (EGTA)
buffer Z	:	50mM KCl and 2mM Tris (hydroxymethyl) aminomethane acetate (Tris-acetate), pH 8.0
buffer Z-1	:	0.1mM KCl, 2mM Tris (hydroxymethyl) aminomethane acetate (Tris-acetate), pH 8.0 and 0.5mM DTT
LISB	:	2mM Tris-acetate, pH 8.0 and 0.5mM DTT
KCl buffer	:	0.1M KCl, 0.15M potassium phosphate, pH 6.5
cin buffer	:	2mM Tris-HCl, pH 8.0, 0.2mM ATP, 0.2mM calcium chloride ($CaCl_2$), and 0.5mM ß-MCE
actin-1 buffer	:	0.1mM KCl, 2mM Tris-HCl, pH 8.0, 0.2mM ATP, 2mM $MgCl_2$, 0.2mM $CaCl_2$ and 0.5mM ß-MCE
buffer A	:	0.30M KCl, 0.15M potassium phosphate, pH 6.5, 20mM EDTA, 5mM $MgCl_2$ and 1mM ATP
buffer B	:	0.6M KCl, 25mM potassium phosphate, pH 6.5, 10mM EDTA and 1mM DTT
buffer C	:	3M KCl, 10mM potassium phosphate, pH 6.5
alpha-actinin buffer	:	5mM Tris-HCl, pH 8.2 and 5mM ß-MCE
benign buffer	:	0.1M KCl, 10mM Tris-HCl, pH 8.0 and 5mM $MgCl_2$

storage buffer	:	50% (v/v) glycerol, 0.1M KCl, 1mM EDTA and 50mM Tris-HCl, pH 7.5
6M urea buffer	:	6M urea, 0.1M KCl, 10mM Tris-HCl, pH 8.0, 5mM $MgCl_2$ and 1mM DTT

APPENDIX 3

Enzyme Activity

One unit of chymotrypsin (3.4.21.1) hydrolyzes 1 μmol of benzoyl-L-tyrosine ethyl ester (BTEE)/min at 25°C, pH 7.8.

One unit of CANP (Calpain - 3.4.22.17) hydrolyzes 1 μg of casein / ml / 1/2 hr at 25°C.